TEXAS REPORTS on BIOLOGY and MEDICINE

VOLUME 19, NUMBER 3 FALL, 1961

Copyright 1961 by the Board of Regents of The University of Texas
Annual subscription $4.00 payable to the Medical Branch Library

Contents

GENERAL AND REVIEW ARTICLES AND ABSTRACTS — PAGE

Proprioceptive Innervation of Striated Muscles in the Territory of Cranial Nerves. *Hiroshi Hosokawa* 405

Cancer of the Lung 1930 to 1960: A Review. *R. H. Rigdon and H. Kirchoff* 465

Abstracts of Papers, Texas Branch, Society of American Bacteriologists, Spring 1961 514

TECHNICAL REPORTS

Some Comparative Effects of Smoked Paper, Tobacco and Cigarettes on Chromosomes *In Vitro*. *Akio Awa, Yasushi Ohnuki, and C. M. Pomerat* 518

The Effect of Guanidine (1) On Experimental Poliomyelitis Induced by Oral Administration of Virus to Cynomolgus Monkeys; (2) On Naturally Occurring Enteroviruses of Cynomolgus Monkeys. *Julio G. Barrera-Oro and J. L. Melnick* 529

Fasting and Small Intestinal Transit. *Ivan E. Danhof* 540

Submicroscopic Morphology of Avian Neoplasms V. Studies on Nephroblastoma. *L. Dmochowski, C. E. Grey, B. R. Burmester, and W. G. Walter* 545

The Use of a Mouse Oral LD50 to Evaluate the Immunogenicity of Trichinella Metabolic Antigens. *Adam Ewert and Leroy J. Olson* 580

Mixed Viral Infections: Interactions between Coxsackie Virus and Poliovirus in Mice and Tissue Cultures. *S. Halperen and S. E. Sulkin* 585

Cytological Studies of Tumors, XXXVI. Alterations of Cell Populations in the MTK-Sarcoma II After Some Experimental Procedures. *Y. Matano and S. Makino* 613

Rapid Progressive Coma in Unsuspected Intracranial Meningiomas. *A. Pineda and J. E. Coe* 625

Enzymes of the Kidney of the Normal and Hypertensive Rat. *M. M. Rahman, A. Grollman, and Lester Packer* 633

Effect of Morphine on Pituitary Cytology and Gonadotrophic Levels in the Rat. *Edward G. Rennels* 646

Chest X-Ray Findings in Early Disseminated *Lupus Erythematosus*. *Edwin G. Troutman and Daniel L. Creson* 658

An Investigation of Intrinsic Sites of Demineralization in Bone: A Microradiographic Study. *G. P. Vose* 676

Stabilization of Poliovirus by Cations. *Craig Wallis and Joseph L. Melnick* 683

Catonic Inactivation of DNA Vacuolating Virus in RNA Poliovirus Suspensions. *Craig Wallis and Joseph L. Melnick* 701

A New Mouse Strain Susceptible to Mammary Cancer. *Alfred Taylor and George F. McKenna* 706

Calling Attention To: 709

FOUNDED BY CHAUNCEY D. LEAKE, 1943

EDITOR: JOE A. BASS

EDITORIAL BOARD

GEORGE A. EMERSON, LEONARD C. HARRIS, GEORGE R. HERRMANN, HARRY LEVINE, WIKTOR W. NOWINSKI, EARL B. RITCHIE, JOHN G. SINCLAIR

GENERAL AND REVIEW ARTICLES AND ABSTRACTS

PROPRIOCEPTIVE INNERVATION OF STRIATED MUSCLES IN THE TERRITORY OF CRANIAL NERVES

HIROSHI HOSOKAWA*

It is generally accepted that the skeletal muscles are supplied not only with motor and autonomic innervation but also with sensory nerves, which, by transmitting proprioceptive sensation, play an important role in the mechanism of the stretch-reflex. As regards the morphological aspects of proprioception, the structure of muscle and tendon spindles, representing the principal proprioceptive nerve endings, have been clearly presented, the peripheral course of their nerve fibers examined, their ganglion cells located in the spinal ganglia, their central processes traced through the dorsal roots into the spinal cord, and their central connections have been shown to pass through the dorsal fasciculus and medial lemniscus to the thalamus and further to the general sensory area of the cerebral cortex.

On the other hand, our knowledge about sensory innervation of striated muscles in the territory of cranial nerves is, excepting in some muscles, so poor that we are not even sure whether or not these muscles are really supplied with afferent innervation.

There are several reasons for the delay of knowledge in the cranial nerve regions.

1) In general no typical sensory nerve endings are developed in the muscles in question. Typical muscle and tendon spindles are often absent and only atypical, rather primitive types of endings are found, which are variant in shape from one animal to another.

2) Since the cranial nerves are not separated into the ventral and dorsal roots, the proof of nature of nerve endings by cutting the motor or sensory component separately is not possible.

3) The fact that the autonomic component, which forms a somewhat independent sympathetic trunk in the spinal region, is more intimately mixed in cranial nerves, makes the situation more confused.

* From the Department of Anatomy, University of Tokyo School of Medicine, Hongo, Tokyo, Japan. Submitted for publication April 10, 1961.

4) The branching and anastomosis in the peripheral course of cranial nerves are so variable among different species of animals, that the results obtained in one animal do not necessarily apply in other animals.
5) As seen in the case of the mesencephalic nucleus of trigeminal nerve, the sensory nerve cells of cranial nerves are not necessarily located outside the brain stem. Therefore, even when the severance of the root of one nerve results in the complete degeration of all nerve endings in one muscle, it does not always prove the lack of sensory endings in that muscle.
6) Further, there is a possibility that the striated muscles in the territory of cranial nerves are originally poor in proprioceptive function. If so, we are, in addition to above mentioned difficulties, faced with the difficulty of searching for receptors which are quantitatively scarce.

Some authors insist that proprioception has an important physiological meaning only in those muscles which support the body weight against the gravity. Others explain that the proprioceptive function is substituted in the cranial nerves by other kinds of sensation such as vision for the eye muscles, audition for the tympanic muscles, gustatory or tactile sensation for the masticatory and tongue muscles.

But in the eye muscles, which had been regarded for a long time as lacking in muscle spindles, many spindles have been recently found and described. Also the masticatory muscles are well known to be furnished with distinct afferent innervation. So it is clear that previous work and opinions are not always trustworthy.

We need many more studies and knowledge about the afferent innervation of the muscles in the territory of cranial nerves. In the following, previous works will be reviewed and discussed, in order to make them useful for future advancement.[1]

I. Sensory Innervation of the Extrinsic Eye Muscles

According to Sherrington (1918), who attached great importance to the proprioception of eye muscles, the stereovisual sensation is completed only by co-operation between vision in the retina, audition in the labyrinth, and proprioception in the

[1] Among many books of neuroanatomy referring more or less to the proprioception problem of cranial nerves, Brodal's "Neurological Anatomy" ('48) presented a pertinent and comprehensive survey of the literature.

extrinsic eye muscles. But the human ocular muscles were regarded for a long time as lacking in sensory innervation, for it was general opinion that those muscles were not provided with muscle and tendon spindles. Also the supposition that the visual sensation might substitute for proprioception of the eye muscles undoubtedly helped this general conception to grow.

As mentioned above, many muscle spindles were recently found in the human extrinsic eye muscles and the former general opinion was jeopardized. Now that the sensory endings have been demonstrated, the peripheral course of nerve fibers, the location of ganglion cells, and the central connecting paths should be examined and determined. However, there is still no agreement as to answers to these questions.

1. *Sensory nerve endings in the extrinsic eye muscles.*

Apart from sensory nerve endings in the skeletal muscles in general, which have been studied by many authors with various kinds of staining methods and classified into many types, those endings reported in the ocular muscles of man and animals may be classified into following groups:

(a) *Muscle spindle* or *neuromuscular spindle*

The muscle spindle, of which the structure and function have been studied by Kühne (1863-a, b; 1864), Kerschner (1888-a, b; 1893), Ruffini (1897, 1898), Sherrington (1894), Huber & DeWitt (1898), Dogiel (1902), Tello (1922), Hines (1927), Hinsey (1927), Barker (1948), Tiegs (1953), Seto (1957), Boyd (1958), etc., represents the most typical sensory end apparatus common in striated muscles of all vertebrates higher than fishes. Electron microscopic structure of the spindle in the rat's lumbrical muscle was recently studied by Merrillees (1960).

In man there were several muscles which were regarded as lacking in spindles. Those exceptional muscles comprise the extrinsic eye muscles, facial and auricular muscles, pharyngeal and laryngeal muscles, muscles of oesophagues, diaphragma, m. styloglossus, m. digastricus, masticatory muscles, m. ishiocavernosus and m. bulbocavernosus. It is probably noteworthy that the majority of these muscles belong to the territory of cranial nerves.

As regards the human extrinsic eye muscles, Batten (1897), Baum (1900) and many other authors looked for muscle spindles

in vain and concluded that they were absent in those muscles. In the eye muscles of animals, a few authors such as Crevatin (1901) and Dogiel (1906-b) reported the presence of spindle-like structures. Then Cilimbaris (1910) described the detailed structure of muscle spindles in the sheep's eye muscles, and further ascertained by comparative anatomical study that the spindles are present in the eye muscles of the deer, sheep, goat, ox and wild boar, while they are absent in the horse, pig, dog, cat, fox, rabbit and rat. But according to Sutton (1915), who studied the development of muscle spindles in the pig's eye muscles, spiral nerve endings were observed in this animal near the end of fetal life.

Then, rather unexpectedly, muscle spindles or spindle-like structures were found also in the human eye muscles by Daniel (1946) and Sunderland (1949), as well as by Cooper and Daniel (1949). Thereafter many authors obtained the same result (Merrillees, Sunderland & Hayhow, 1950; Wolter, 1952, 1953, 1955; Cooper, Daniel & Whitteridge, 1955; Voss, 1957; Okamoto, 1957; Inoue, 1958; Wolff, 1958, etc.). According to these authors, muscle spindles in the human eye muscles measure 500–1000μ in length, 50–100μ in thickness, and the average number of intrafusal fibers of Weissmann, which measure some 10–20μ in thickness, is four to five. As the total number of spindles in each muscle is from 30 to 70, which is considerable, it is rather difficult to understand how they had escaped the keen search of previous authors. It might have been, so to say, a kind of the blind-spot phenomenon.

Noteworthily several Japanese authors reported muscle spindles in the human eye muscles previously (Adachi, 1911; Hamada, 1928; Yokomatsu, 1932). (Adachi was cited from Kiyono, 1911). In this relation the work of Hatano (1925) and Nakayama (1911) should be remembered, too. Hatano found a spindle in both the ishiocavernosus and bulbocavernosus muscles, while Nakayama described spindles in the laryngeal and oesophageal muscles. Also the paper of Siemerling (1888) noted here, who incidentally described spindle-like structures in an atrophic human inferior rectus muscle, which was compressed by a gumma.

Nerve terminations in the muscle spindle in general were classified by Ruffini (1898) into three types, the annulo-spiral

ending (terminaison annulo-spirales), flower-spray ending (terminaison en forme de flours) and the plate-like ending (terminaison en plaques). The former two types of ending are supplied by thick nerve fibers (δ-fibers of A group) and are thought to be sensory, while the last one receives thin fibers (γ-fiber of A group) and regarded as motor in nature.

Concerning the location within the spindle, the annulo-spiral ending lies in the middle portion and the flower-spray endings are on one or both sides of the former, whereas the plate-like endings are found near the extremities of the spindle. Recently the functional differentiation among these different types of nerve endings were subjected to studies by physiologists (Wakabayashi, 1958; Hongo, et al. 1959; Shimazu, 1959, etc.).

In the muscle spindles of sheep's eye muscles Cilimbaris (1910) identified sensory and motor endings also, and the present author obtained similar results in the spindles of goat's eye muscles (Fig. 1). In the human ocular muscles, however, there

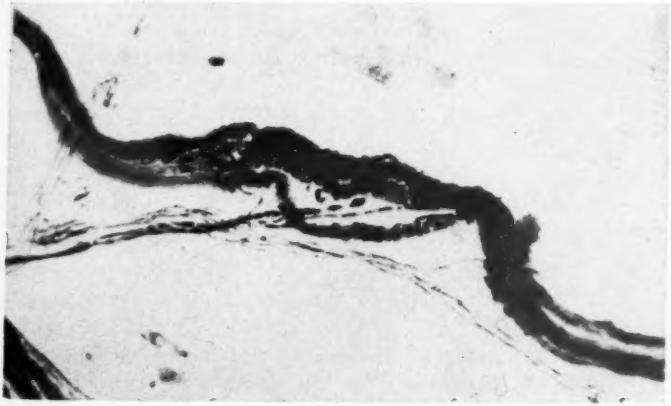


FIG. 1. Muscle spindle, isolated from the extrinsic eye muscle of goat. Gold chloride stain. (Hosokawa)

are often such atypical muscle spindles which are supplied only with the annulo-spiral endings. They were found for the first time by Daniel (1946) and were dscribed as "spiral nerve endings." According to Okamoto (1957), these spiral endings usually entwine around a single muscle fiber which is almost of the

same size as ordinary, extrafusal fibers, and the connective tissue capsule is very often absent. So, strictly speaking, they may not be called 'muscle spindle,' representing rather a simple or primitive type of sensory end apparatus. Needless to say, besides the spiral endings, typical muscle spindles have been also found in the human eye muscles.

Muscle spindles are said to be located mainly in the proximal and distal one-third of each eye muscle, while they are rarely found in the middle part (Merrillees, Sunderland & Hayhow, 1950; Voss, 1957). According to Inoue (1958), however, there are a considerable number of typical spindles also in the middle one-third (Fig. 2, Table 1). Cooper, Daniel and Whitteridge

TABLE 1

Distribution of muscle spindles in the human extrinsic eye muscles
(Inoue, '58)

	Proximal one-third	Transition zone	Middle one-third	Transition zone	Distal one-third	Total number
M. rectus superior	26	7	11	4	19	67
M. rectus inferior	27	4	11	0	6	48
M. rectus medialis	22	1	12	0	12	47
M. rectus lateralis	13	0	10	0	8	31
M. obliquus superior	15	0	9	4	13	41
M. obliquus inferior	13	1	6	0	6	26
M. rectus lateralis (suppl.)	7	1	5	0	7	20

(1955) showed diagrammatically the distribution of various kinds of sensory endings in the human rectus oculi muscle (Fig. 3). According to them, the muscle spindles are mainly located in the proximal one-third, while the motor end-plates occupy the middle one-third. Spiral endings are distributed mainly in the area just distal to the zone of motor plates.

(b) *Tendon spindle* or *neurotendinal organ of Golgi*

Golgi, the discoverer of the tendon spindle or "organo nervoso terminale musculo-tendineo" (1800-b, 1894-a), ascertained that this organ is widely distributed in the muscles of vertebrates higher than fishes. He noticed also that the tendon spindle is absent in the eye muscles.

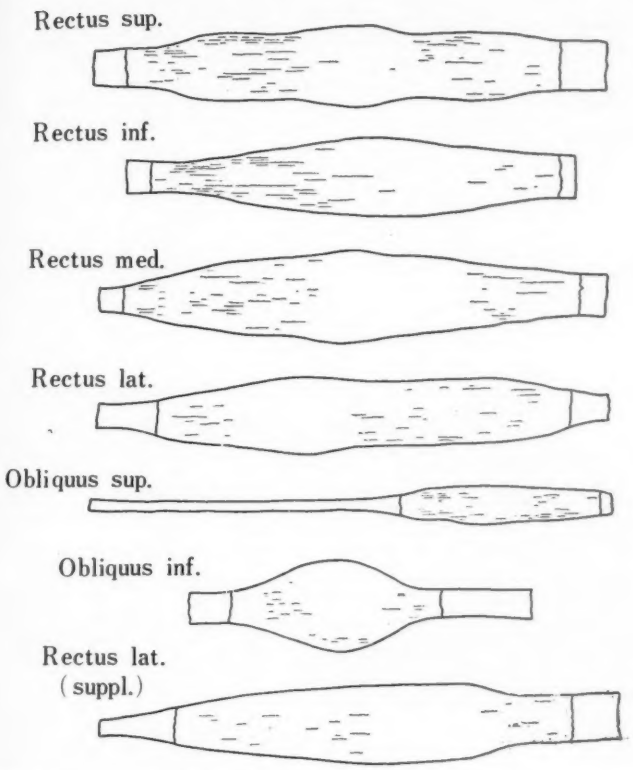


FIG. 2. Distribution of muscle spindles in the human extrinsic eye muscles. (Inoue, 1958)

Although some authors such as Marchi (1882), Huber (1900), Crevatin (1901) and Dogiel (1906-b) mentioned tendon spindles in the eye muscles, they were mostly not typical spindles but were merely spindle-like nerve terminations found in the musculo-tendinous transition zone.

A typical tendon spindle is represented by complicated arboreal branching of fairly thick nerve fibers located in a swelling of fused tendon fibers (Huber & DeWitt, 1900). The terminals of nerve fibers usually show leaf-like enlargements (Fig. 4). The spindle is enclosed in a conective tissue capsule, which is well

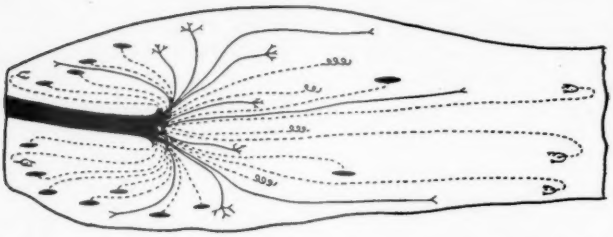


FIG. 3. Diagram to show the distribution of sensory nerve endings in the human rectus oculi muscle. (Cooper, Daniel & Witteridge, 1955) The muscle spindles are most numerous in the proximal third of the muscle, while most of the motor nerve endings are in the middle third. The simple spiral endings are located just distal to the zone of motor endings. The tendon endings are seen in both the origin and insertion of the muscle.

FIG. 4. Nerve terminations in the tendon spindle. A flexor muscle of cat's fore limb. Gold chloride stain. (Hosokawa)

developed in birds and mammals. In the amphibia and reptilia, however, the connective tissue sheath is thin and often absent.

Typical tendon spindles found in the eye muscles were reported only by Dogiel (1906-b), in the ox. Spindle-like structures described by Huber (1900), *et al.* are formed in such a way that thick nerve fibers run toward the tendon and, taking a recurrent course at the transition zone, and ramifying into branchlets, surround each tendon or muscle fiber in a palisade fashion. Although Huber (1900) mentioned thin connective tissue sheaths, no other author referred to them.

It is noteworthy that these atypical tendon spindle-like nerve terminations are fairly widely distributed in the eye muscles of man and animals such as the monkey, dog, cat, horse, pig, rabbit, etc., though they are absent in the m. retractor bulbi, the striated muscle common in the animal orbit.

Judging from the shape of nerve terminals, it is agreed that they are receptors for afferent impulses. Sherrington (1897) as well as Tozer and Sherrington (1910) regarded them as substitutors for muscle spindles, which were thought to be absent in the eye muscles, and used them as the guide to determine the peripheral course of sensory nerve fibers (see below).

According to Hinsey (1927), Giacomini (1898) described basket-like nerve endings (terminaison en panier) surrounding the ends of muscle fibers of fishes and urodeles and regarded them as sensory end apparatus more primitive than the muscle or tendon spindle. It is likely that these "terminaison en panier" have some community with the atypical tendon spindles of the eye muscles.

(c) *Grape-like ending*

Nerve terminations of this category which have been called by various names such as "terminaison en grappe" (Tschiriew, 1879), "doldenförmige Endigung" (Bremer, 1882, 1883), "terminazioni a grappolo" (Ciaccio, 1891; Giacomini, 1898, etc.), "atypische motorische Endigung" (Retzius, 1892), "traubenförmige Endigungen," etc. are not represented by a single type but comprise a group of nerve terminals with shapes of considerable variation. They were originally described by Tschiriew (1879) in the muscles of a reptile (Tropidonotus natrix) and regarded as young, immature form of the motor end plates. He called them "terminaison en grappe," because their shape reminded of a bunch of unripe grapes, of which each fruit is small and apart from each other. When they were mature, he probably thought, the whole bunch would be filled with large fruits, resulting in the typical motor end-plate. According to this interpretation, it is natural that there are sometimes transitional forms which are rather difficult to discriminate from the typical motor plates.

Retzius (1892) saw similar nerve endings in the rabbit's eye muscles and took them, instead of immature forms, for atypical motor endings (Fig. 5). Some others thought also that they might

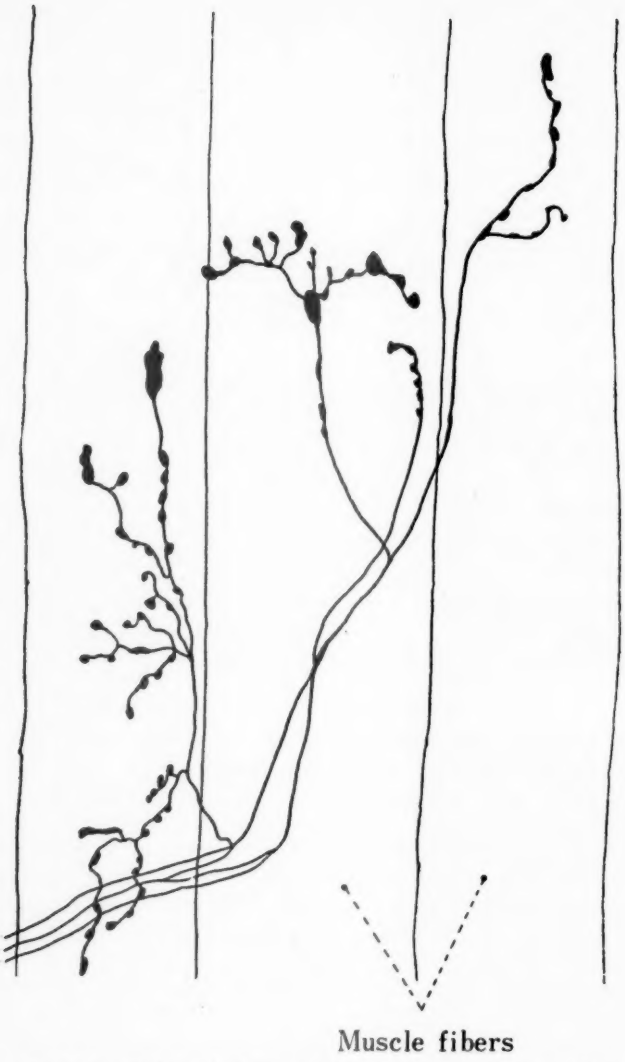


FIG. 5. So-called "atypical motor endings" of Retzius. (Retzius, 1892)

be motor in nature (Ciaccio, 1891; Thanhoffer, 1892; Perroncito, 1902; Garven, 1925; Cogan, 1948, etc.).

When the characteristic separation of each terminal branchlet of the grape-like ending is accented, we obtain various modes of nerve termination, some of which resemble very much the flower-spray ending in the muscle spindle or the nerve terminals in the tendon spindle (Fig. 6). So it is reasonable that some

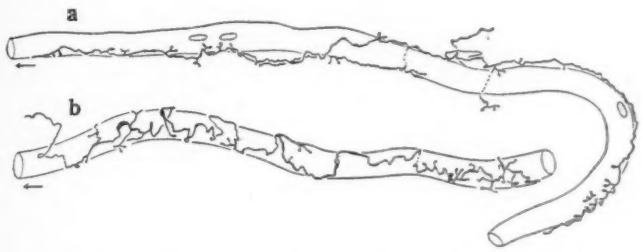


FIG. 6. Sensory nerve endings in the extraocular muscle fiber of rabbit (a) and kitten (b) representing extremely dissociated grape-like endings. Methylene blue supravital stain. Arrows show the distal direction. (Hosokawa)

authors took them for sensory endings (Bremer, 1882, 1883; Kerschner, 1888; Giacomini, 1898; Huber, 1899; Dogiel, 1906; Kulchitsky, 1925; Hinsey, 1927; Woollard, 1931; Hines, 1931, etc.).

It is easy to understand, therefore, why grape-like ending has been used as a collective noun to comprise all the atypical nerve endings except the typical motor or sensory ones. It is probable that some grape-like endings are motor and others sensory, while there are still others which may be, as Boeke (1927) said, autonomic in nature.

It is seldom easy to decide morphologically the nature of nerve endings in the muscle. It is stated that: while motor endings are located under the sarcolemma (hypolemmal) and the sole plate (Doyer's hillock) is formed by the accumulation of granular substance, sensory endings are situated on the sarcolemma (epilemmal) and lack in the sole plate. Judgment of the individual ending is, however, often very difficult. Histochemical differentiation between the motor and sensory nerve endings would be highly evaluated, if some adequate method is devised.

(d) *Sensory endings in the interstitial connective tissue*

There have been reported several kinds of sensory endings in the interstitium of striated muscles. First of all, Sherrington (1894) mentioned "free endings" in the muscle, the existence of which has not been confirmed yet. Dogiel (1906) described tree-like branching ("baumförmige Verzweigungen") in the eye muscles of man and animals. Afterwards Kulchitsky (1924) also referred to similar structures. Since they did not mention a connective tissue capsule, it is uncertain whether those endings were naked or furnished with the capsule.

Concerning a sensory end apparatus enclosed by the connective tissue capsule, the following items are to be mentioned.

(i) Pacinian corpuscles. Lamellar corpuscles of Vater-Pacini have been reported by several authors in the interstitium of striated muscles (Golgi, 1880; Kerschner, 1888; Cattaneo, 1888; Ruffini, 1897; Horsley(1897; Stöhr, 1928; Azuma, 1951). The author has seen them in the tendon of a flexor muscle of the cat's fore limb (Fig. 7). However, nobody has encountered this apparatus in the eye muscles.

(ii) Corpuscles of Golgi-Mazzoni. Lamellar corpuscles of smaller size found for the first time by Mazzoni (1890) were described by Dogiel (1906) in the eye muscles of man and animals. Furthermore, Shimada (1956) and Nozaki (1956) observed a sensory end apparatus of this kind in the muscles of lower extremity of the human fetus.

(iii) Corpuscles of Ruffini. This kind of sensory organ discovered by Ruffini (1894), although encountered in the ordinary muscles and tendons, has not yet been described in the eye muscles.

(iv) Others. According to Wolter (1955), who studied the sensory nerve endings in the human eye muscles by means of silver technique, sensory nerve terminations in the interstitium are classified into following types: flower-bud-like endplates, arboreal terminations, brush-like end structures, sensory spools, and sensory end-bulbs. Very often clusters of cells or nuclei were found around the nerve terminals, and all of those endings were enclosed by connective tissue capsules. Several authors mentioned terminal boutons on the blood vessels in the interstitium. They probably represent the autonomic end apparatus.

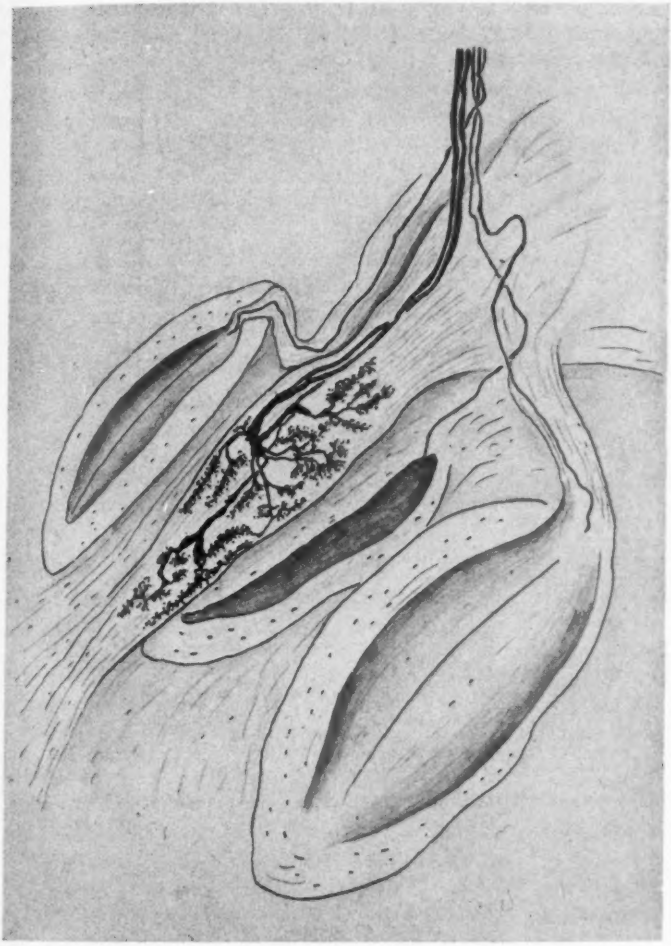


FIG. 7. Tendon spindle and Pacinian corpuscle-like end-organs. Cat's fore limb. Gold chloride stain. (Hosokawa)

2. *Peripheral course of nerve fibers for transmitting afferent impulses from the extrinsic eye muscles.*

It is quite reasonable to assume that the first branch of the trigeminal nerve, n. ophthalmicus, may be responsible for trans-

mitting the proprioceptive sensation of the extrinsic eye muscles, because all the other nerves concerned, such as the third, fourth and sixth nerves, are thought to be purely efferent in nature. Furthermore, the anastomoses between the ophthalmic nerve and those efferent nerves in the depth of the orbit make it probable that proprioceptive fibers, which originally belong to the ophthalmic nerve, run through these anastomoses into the third, fourth, or sixth nerve so as to supply the muscles together with motor fibers.

These anastomoses were described by Schwalbe (1881), Stibbe (1929), Sinclair (1958) and others in human specimens (Fig. 8). They are located usually around the superior orbital fissure

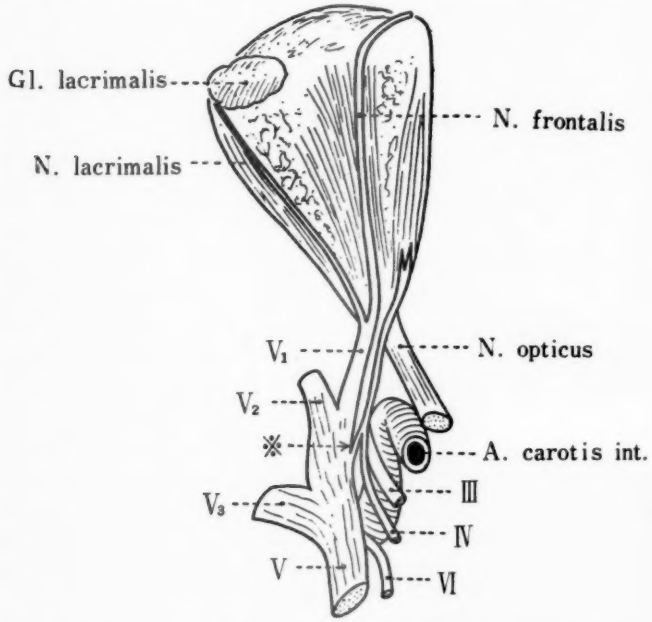


FIG. 8. An anastomosis between the trigeminal and trochlear nerve roots of man. (※). (Hosokawa)

or even in the walls of the cavernous sinus, although they are not constant, especially those going to the fourth or sixth nerve. As regards other mammals, Kiss (1935) reported similar anasto-

moses in the antelope, and Omoto (1959) demonstrated a communication between the frontal and trochlear nerve in the rabbit.

According to Winckler (1937, 1956), who studied the pig, sheep, goat, ox, rabbit, deer, etc., each rectus and oblique muscle receives, in addition to motor nerve, direct branchlets from the trigeminal nerve. The present writer (Hosokawa, 1951) noted that, in the fetus of the sperm whale, some twigs of the fifth nerve supplied the eye muscles directly or after joining the motor branches (Fig. 9).

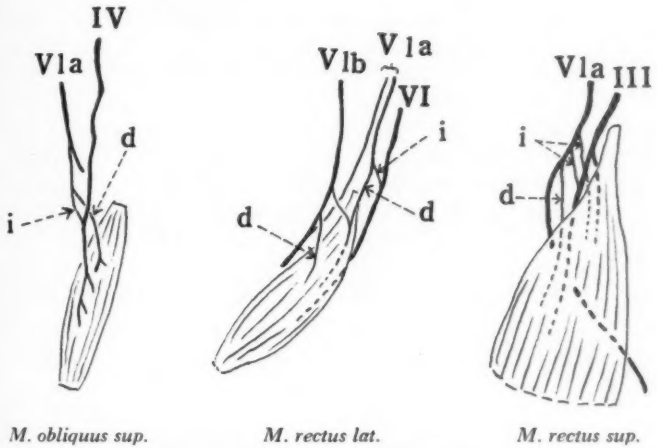


FIG. 9. Sensory innervation of extrinsic eye muscles of a fetus of sperm whale. (Hosokawa, 1951) d: direct insertion. i: indirect insertion.

Another possibility for a peripheral course of ocular proprioception, which appears to be rather contrary to the trigeminal nerve theory, was proposed by Sherrington (1894, 1897, 1898, 1918) as well as by Tozer and Sherrington (1910). After having proved the sensory innervation of striated muscles in the spinal nerve region, they directed attention to the extrinsic eye muscles. At first Sherrington sectioned the trigeminal and optic nerve in monkeys and cats, so as to examine the possible changes in the ocular movements and tonus. However, as he saw no remarkable changes, he assumed that sensory innervation of extrinsic eye muscles must take place through the motor nerves themselves. Then, after sectioning roots of the third, fourth and sixth

nerves at the exit from the brain stem, he found that all the nerve fibers in the peripheral stumps as well as in the innervated muscles were, excepting a small number of thin, apparently autonomic fibers, completely degenerated. Regarding the nerve endings, not only the motor end plates but also the atypical tendon spindles described in the preceding paragraph disappeared completely, which implied that his assumption was correct.

Similar experiments have been carried out repeatedly by Hines (1931) and Fukuda (1956, 1957, 1958) in the rabbit and by Tarkhan (1934) in the cat. In all instances Sherrington's conclusion was confirmed. In this relation it should also be noted that Abd-El-Malek (1938) reported the rise of blood pressure by stimulating the central stump of the dog's third, fourth and sixth nerve.

According to Hines (1931), a few thin nerve fibers left intact in her above mentioned experiments underwent degeneration by the removal of the superior cervical ganglion. She located the terminal endings of these thin fibers in the walls of small blood vessels, while Boeke (1927) described autonomic nerve endings on the muscle fibers themselves. (Many works dealing with the autonomic innervation of striated muscles have been published. In this review, however, the author omits all of them, since they are not directly connected with the sensory innervation of muscles.)

Needless to say, the ophthalmic nerve was cut as the control experiment in some of the above mentioned studies (Tozer & Sherrington, Fukuda). At that time they did not see any degenerative changes of nerve endings in the eye muscles, and the atypical spindle-like apparatus was also shown to be intact.

This result seems to weaken further the possibility of participation of the trigeminal nerve in the proprioceptive innervation of eye muscles. But Tozer and Sherrington (1910), in the specimen of which the ophthalmic nerve was sectioned just distally to the semilunar ganglion, recognized a few degenerated nerve fibers in the peripheral part of the third, fourth and sixth nerve. Although it was not ascertained by serial sections, these degenerated fibers might have been derived from the fifth nerve through anastomoses between the nerve roots. Tozer and Sherrington did not make clear the destination of these degenerated fibers.

Now it is shown that, contrary to the common conception, the third, fourth and sixth nerve may not be purely motor in nature, but possibly contain some afferent fibers to transmit proprioceptive impulses from the extrinsic eye muscles. Does this mean that the common conception, which makes the ophthalmic nerve responsible for this task, is completely erroneous?

Rather recently the trigeminal nerve theory reappeared in an unexpected way in the work of Cooper, Daniel and Whitteridge (1949, 1950, 1951, 1953, 1955). They endeavored to trace electro-physiologically the centripetal course of sensory impulses caused by stretching the goat's extrinsic eye muscles. While the action potential was recorded at first in the oculomotor nucleus (1950, 1951), they reported afterwards that the nerve cells of low threshold receptors such as the muscle spindle might be located in the semilunar ganglion or the nucleus of the mesencephalic trigeminal tract (1953-a). They then reached the elaborate conclusion that the receptors in the extrinsic eye muscles of the goat should be divided into the high and low threshold groups, of which the former are related to the motor nerve roots and nuclei, while the latter are intimately connected to trigeminal elements (1955).

In this way two contrary possibilities of the peripheral course for transmitting proprioceptive impulses from the eye muscles appear to have been reconciled within each other. Needless to say, the significance of muscular branches of the ophthalmic nerve as well as of the anastomoses between this nerve and motor nerve roots has increased together with the revival of the trigeminal nerve theory.

As a matter of fact, Whitteridge (1955) recorded afferent action potential in the branchlet of the ophthalmic nerve supplying the superior oblique muscle of goat. The same might hold good for similar branches or anastomoses in man, baboon and cat (Cooper, Daniel & Whitteridge, 1955). According to Cooper and Fillenz (1952, 1955), who studied the extrinsic eye muscles of the cat and monkey electro-physiologically, afferent potential was recorded only in the peripheral root of the oculomotor nerve. It should be noted that eye muscles of the cat and monkey are lacking in muscle spindles, while there are plentiful in the goat's eye muscles.

3. *Ganglion cells in the ocular motor nerve roots.*

As described above, Sherrington and others have shown that the third, fourth and sixth nerve are not purely motor in nature but have at least some of the afferent nerve fibers from the extrinsic eye muscles. The localization of ganglion cells of these fibers is, however, not yet known. Some people assume that the cells in question must be represented by the ganglion cells scattered along the peripheral roots of the ocular motor nerves.

Thomsen (1887) was the first to notice some peculiar features such as degenerated nerve cells in the roots of third, fourth and sixth nerves of a human newborn. Similar observations were made by Gaskel (1889) in the third, fourth, sixth, eighth, and ninth nerve, and Golgi (1893) also described ganglion cells in the trochlear nerve root proximally to the decussation.

Tozer (1912) carefully examined these cells in the monkey and found on the average 21 cells (0–74) in the third and 30 cells (0–78) in the sixth, while he saw none in the fourth nerve root. Besides, he noticed some cells in the third nerve of the dove. A fish (Gadus vireus) also showed a considerable number of cells in the third (80), fourth (30), and sixth nerve (32–39). But Tozer apparently hesitated to regard them as sensory cells related to the extrinsic eye muscles.

Nicholls (1915) had a similar experience and Nicholson (1924) paid attention to the fact that the number of these cells roughly correspond to that of thin nerve fibers which were left intact in the experiment of Tozer and Sherrington (1910) after sectioning the root proximally. Nicholson counted 44 cells in the third as well as 30 cells in the sixth nerve of a human embryo at term. These cells were medium-sized, ranging from 20 to 35μ in diameter.

Pankratz (1930) found some nerve cells in the ocular motor nerves of the toadfish (Opsanus tau). Similar cells were described by Windle (1932) in the trochlear nerve of the cat's embryo just proximally to the decussation. Later on, Pearson (1943-a, 1944) also noticed some cells in the third and fourth nerve of the human embryo, and Hakamada (1943) encountered a few, medium-sized nerve cells enclosed by satellite cells in the baboon's oculomotor nerve root.

According to Fukuda (1955–1958), who examined these gan-

glion cells in the rabbit precisely and carefully, the cells were found in the third and sixth nerves of all the fourteen animals studied, the average number of cells being 36 (11–132) and 43 (5–169) respectively. On the other hand, the fourth nerve showed, only in one case, a group of about one hundred nerve cells. Most of these cells were located along the nerve trunks in the orbit, especially at the sites where the muscular branches were given off. The cells varied in size from 16 to 43μ, the majority being between 25 and 32μ.

In order to determine the nature of these cells Fukuda (1956, 1958) examined the pattern of Nissl bodies as well as the effect of section of nerve roots. He was inclined to the opinion that the cells in question are not the proprioceptive ganglion cells but belong to the autonomic nervous system, representing possibly cells of the accessory ciliary ganglia.

More recently Rogers (1957) studied the cells in the ocular motor nerve roots of chick embryos in various stages of development (5–17 days of incubation). He was convinced that these cells were responsible for the proprioceptive innervation of extrinsic eye muscles. According to his hypothesis, those cells originated from the neutral crest and were taken into the brain stem as the mesencephalic nucleus of the fifth nerve (see below).

Sinclair (1958) reported cell clusters in the fourth nerve of a human embryo of 20 mm. as well as of a porpoise embryo of 25 mm. (Prodelphinus caeruleo-albus). On the latter the cells formed a ganglion 1.5 mm. long which contained hundreds of large cells. The same author paid special attention to the fact that a count of nerve fibers of the fourth nerve in the adult human outside the brain stem was nearly twice as much as that of the fascicles before decussation. He could not determine the source or nature of these extra fibers, whereas he noted a large number of small fibers entering just before the decussation.

Sano (1941) studied the trochlear nucleus of various kinds of mammals and found that the posterior part of this nucleus is very often separated from the main mass. This cell cluster, which may be called posterior trochlear nucleus, is located near the caudal end of the main nucleus in man, rabbit and cat, while it is displaced far caudally to the middle of the intracerebral course of this nerve in the baboon. In the adult human brain, Sinclair

(1958) reported that the most caudal motor cells of the fourth nerve lie almost at the level of the decussation and their fibers sweep independently into the fascicles at various levels.)

Supplement. Ganglion cells in the other cranial motor nerve roots.

Ganglion cells have been reported from time to time also in the cranial nerve roots which have nothing to do with the movements of eye ball.

(i) Trigeminal nerve. In the trigeminal nerve root of man, rabbit, dog, cat, ox, pig, etc. Peters (1935) found accumulations of nerve cells sometimes reaching more than one hundred in number. Probably most of these cells were of the same kind as those of the semilunar ganglion. However, the significant fact is that he saw some cell clusters also in the portio minor. Nerve cells in the portio minor had been described by Allen (1925) in the cat, too.

(ii) Facial and glossopharyngeal nerve. As mentioned above, Gaskel (1889) recognized nerve cells in the root of the seventh and ninth nerve of human material. Large numbers of migrating nerve cells may be seen on all branchial nerves in the foetal dolphin (37 mm.).

(iii) Vagus nerve. In the Japanese monkey and baboon Hakamada (1943) found remarkable groups of nerve cells in the vagus root at the site where it penetrated through the arachnoidea as well as inside the subarachnoideal space. Judging from the shape, position and number of these cells, he regarded them as equivalent to those of the jugular ganglion. Besides, nerve cells are known to be scattered from time to time along the vagus trunk.

(iv) Accessory nerve. A small ganglion of large cells was described by Hakamada (1943) in the bulbar root of the baboon's accessory nerve. Pearson and O'Neil (1946) happened to observe bipolar cells in the spinal roots of the same nerve in the mouse embryo. Other reports will be mentioned in Section IV, below.

(v) Hypoglossal nerve. As described later in Section VII, ganglion cells in the twelfth nerve root have been reported by Langworthy (1924) in the cat, Pearson (1942-b, 1945-a) in the human embryo and adult, and by Tarkhan and Abd-El-Malek (1950) in man, rabbit, cat and rat.

(vi) Ventral roots of spinal nerves. Windle (1931-c) found

a few nerve cells in the spinal ventral roots of the cat, dog, guinea pig and man. According to him Piolti examined one ventral root of the thoracic and lumbar nerves respectively and saw considerable numbers of nerve cells, which looked like sensory cells.

(vii) Dorsal roots of spinal nerves. Youngstrom (1944) saw bipolar cells among the dorsal root fibers within the spinal cord of a human embryo. Similar aberrant ganglion cells were studied also by Duncan and Crocker (1939).

4. *Where is the nucleus which receives proprioceptive impulses from the extrinsic eye muscles?*

Two possibilities of the peripheral course of proprioceptive nerve fibers from extraocular muscles were discussed in the preceding section. Two theories have been proposed concerning the nucleus which receives these proprioceptive impulses, but the final decision has not been given.

(a) Authors who accept the close relationship between the ocular proprioceptive fibers and the trigeminal mesencephalic tract or nucleus.

Following Golgi (1893, 1894-b), who wrote that some fibers of the mesencephalic fifth tract enter the trochlear nerve root, the intimate relationship or continuation of nerve fibers between the mesencephalic tract and ocular motor nerves have been accepted histologically by Weinberg (1928) in various vertebrates, by Windle (1932) in the cat's embryo, by Tarkhan (1934) in the cat as well as by Pearson (1943-a, 1944) in the human embryo, infant and adult.

The last named author wrote further that some cells of the trochlear and oculomotor nucleus in the silver preparations of the human embryo were quite similar to those of the trigeminal mesencephalic nucleus, implying the possibility that these cells might send sensory fibers to the third and fourth nerve roots. Sinclair (1958) could, however, find none of this type of cells in silvered adult human material except along the afferent fascicles.

Degeneration experiments were carried out by Freeman (1925, 1927) and Sheinin (1933). When they removed the whole content of cat's orbit, the mesencephalic nucleus was shown to be partly degenerated and they explained this by the destruction of afferent nerve fibers from the eye muscles. According to Freeman, no degeneration was seen when only the ophthalmic nerve

was cut. Therefore he was led to the conclusion that the afferent fibers from the eye muscles must be transmitted through the ocular motor nerve roots into the mesencephalic fifth nucleus.

Woollard (1931) reported also that the sectioning of the rabbit's third nerve root caused degeneration in the mesencephalic nucleus at the level of the oculomotor nucleus.

Based on these observations and reports, Brodal (1958) was inclined to regard the mesencephalic fifth nucleus as the most possible site for the ocular proprioceptive fibers to terminate, although these histological and experimental studies were still not conclusive nor unanimous. If these degeneration experiments are correct, the ocular proprioceptive nerve fibers in question should be represented by direct continuations or processes of the cells of trigeminal mesencephalic nucleus, a condition ascertained for the afferent nerve fibers from the masticatory muscles (Section II). Electro-physiological evidence for the close association between the eye muscles and mesencephalic nucleus was proposed by Fillenz (1955), who recorded action potential around this nucleus by stretching the inferior oblique muscle of the cat.

(b) Authors who deny the relationship between the ocular proprioception and the trigeminal mesencephalic tract or nucleus.

In his distinguished book on the histology of the nervous system (volume 1, 1909, new print, 1952) Cajal denied the above mentioned statement of Golgi (1893, 1894-b) which accepted the fibrous continuity between the trigeminal mesencephalic tract and ocular motor nerve roots. Exeprimental study of May and Horsley (1910) in the monkey showed that the intracerebral sectioning of the mesencephalic fifth tract did not provoke any degeneration in the third or fourth nerve roots, and Tozer (1912) confirmed in the same animal that the severance of the ocular motor nerves did not lead to degeneration in the mesencephalic tract or nucleus.

These observations, quite contrary to those in the preceding paragraph, were enforced further by a series of reports of Corbin *et al.*, who did detailed and extensive researches anatomically and physiologically in many cats. At first Corbin (1940) destroyed various parts of the mesencephalic tract and nucleus, and, after a lapse of nine days, all the cranial nerves were

stained with osmic acid. Then degenerated nerve fibers were found only in some branches of the trigeminal nerve, which supplied the mastication muscles, palate, gingiva and periodontium. Electro-physiological studies of Corbin and Harrsion (1940) could not record action potential in the mesencephalic tract, when the extrinsic eye muscles were stretched.

Thus Corbin and coworkers doubted the interrelation between the mesencephalic fifth tract and eye muscles. They thought that the degeneration reported by Freeman (1925) and Sheinin (1933) must have been due to the lesion of the anterior ethmoidal nerve, which was shown to contain some fibers continuous with the mesencephalic tract.

In search for the true sensory nucleus for eye muscles, Corbin *et al.* continued their work and were led to an unexpected destination. This was the motor nuclei which give rise to ocular motor nerves such as the third, fourth, and sixth nerve. While Corbin and Oliver (1942) encountered no degeneration in these ocular motor nerve roots in case of sectioning the mesencephalic tract, they recognized that, if the lesion involved the oculomotor or trochlear nucleus, not only degeneration occurred in the nerve fibers of these motor nerve roots but also the grape-like endings in the eye muscles disappeared. Besides, according to the electrophysiological study of Corbin and Harrison (1942), stretching of the eye muscles provoked action potential only in or around the oculomotor or trochlear nucleus.

In this way the rather strange possibility was proposed that the ocular motor nuclei may be, contrary to the common conception, not purely motor in nature but may also contain some sensory elements.

A similar hint had been suggested already by McLean (1926, 1927), who discriminated in the dog's oculomotor nucleus two kinds of cells, of which the smaller ones were regarded as afferent or proprioceptive for the extrinsic eye muscles. In this relation Sinclair's observation (1954) on the human trochlear nucleus must be considered also. According to him, the fibers of the fourth nerve collected fanwise from the nucleus which was composed of medial and lateral groups of cells. He also cited Olszewski (1954) who pictured supratrochlear nuclei segmented to correspond with the motor cells and connected with them.

We have reviewed the two opposite theories or observations on the locality of afferent nucleus for the eye muscles. Do these two possibilities, then, essentially contradict each other? Or is there any way to reconcile these to each other? Although the final solution has not been reached, it may be remembered that some authors have held opinions which might reconcile these contradictory observations to some extent.

As referred to before, Pearson (1943-a, 1944) found in the oculomotor and trochlear nucleus of the human embryo some cells of the mesencephalic fifth nucleus type, and suggested that they might represent the sensory cells for the proprioceptive innervation of the extrinsic eye muscles. Later Rogers (1957), as mentioned in the preceding section, made similar observations in chick embryos of various stages of development and wrote a hypothesis about the gradual displacement of the proprioceptive nerve cells for extrinsic eye muscles. According to his hypothesis, the ocular motor nuclei may comprise, in addition to their original motor cells, displaced elements from the trigeminal mesencephalic nucleus. Thus the double character of the ocular motor nuclei is easy to understand.

A series of papers by Cooper, Daniel and Whitteridge (1951, 1953, 1955) have brought forward another possibility for reconciling the two contrary viewpoints in an unexpected way. As mentioned briefly before, they discriminated high and low threshold receptors in the goat's extrinsic eye muscles, and related the former to the motor nuclei, while the latter, low threshold receptors were regarded as being in association with the trigeminal mesencephalic nucleus. This duplex theory of the proprioceptive innervation of the eye muscles may be worth examining further.

When Cooper *et al.* (1953-b, c) looked for the secondary pathways for the proprioception of eye muscles of the goat, they obtained action potentials in the central tegmental tract, medial longitudinal fasciculus, superior cerebellar peduncle, colliculotegmental fibers as well as in the cortical area near the visual area.

In his report of patients of levator-pterygoid synkinesis, where the elevation of upper eye lid caused mouth opening reflex, Sano (1959) assumed a reflex arc, which originates in the superior

levator muscle and is transmitted to the trigeminal motor nucleus.

II. Sensory Innervation of the Masticatory Muscles

So far as the sensory innervation is concerned, the mastication muscles present the most stable knowledge among the striated muscles in the territory of cranial nerves. Muscle spindles are well developed (Baum, 1900; Gregor, 1904; Freimann, 1954), and it has been confirmed that the afferent nerve fibers run through the portio minor of the trigeminal nerve into the mesencephalic tract, so as to terminate in its nucleus (Johnston, 1909; May and Horsley, 1910; Willems, 1911; Kosaka, 1912; Allen, 1919; Thelander, 1924; Weinberg, 1928; Corbin, 1940; Corbin and Harrison, 1940). Electro-physiological evidence was also presented by Corbin and Harrison (1940) as well as by Cooper, Daniel and Whitteridge (1953-a), who recorded action potentials in this nucleus by stretching the mandibular muscles of the cat and goat respectively.

It is now the generally accepted conception that the mesencephalic fifth nucleus is a mass of cells which belong to the peripheral, primary neuron. In other words, the peripheral nerve fibers in the portio minor are nothing but the dendritic prolongations of nerve cells of the mesencephalic fifth nucleus. So this nucleus corresponds to the semilunar as well as spinal ganglia, although it is located inside the brain stem. In this point this nucleus represents a remarkable exception in the general structure of the nervous system.

This situation was clearly illustrated in Cajal's book (Fig. 393) (Fig. 170 in the textbook of Ransom and Clark, 1951), and was ascertained repeatedly by degeneration experiments. Cajal saw further that short collaterals are given off to the trigeminal motor nucleus. Probably these collaterals (or axons?) form a reflex arc which controls smoothly the action of the masticatory muscles. Cajal, however, had regarded the mesencephalic nucleus as motor in nature. So his interpretation of these collaterals was naturally a little different. According to him, the mesencephalic nucleus was to store the stimuli from higher centers in its large cells for a while and then to release the energy effectively to the

masticatory nucleus and muscles, provoking powerful, coordinated chewing movements of the muscles (Edinger, 1911).

This nucleus had for a long time been thought to be of motor function. At first Stilling (1846), Deiters (1865) and Golgi (1894-b) regarded it as the nucleus of origin of the trochlear nerve. Then it was taken for a motor nucleus of the fifth nerve by many authors such as Henle (1879), Bregmann (1892), Held (1893), Kölliker (1896), Terterjanz (1899), Kure (1899), Obersteiner (1901), van Gehuchten (1906), Cajal (1909), Vogt (1910), Edinger (1911), Mingazzini (1928) etc. They judged so by the above mentioned relation between this nucleus and the peripheral nerve fibers as well as by the fact that the nerve fibers course through the portio minor representing the motor part of the trigeminal nerve.

However, the large and oval shape of these cells, which resemble considerably those of the spinal and semilunar ganglia, is quite unique, or rather unnatural for a motor nucleus. Some authors (Huguenin, 1873; Merkel, 1874; Lewy, Groff and Grant, 1938) even regarded this nucleus in association with autonomic functions.

Later it became accepted generally as a sensory nucleus of the trigeminal nerve (Johnston, 1909; Willems, 1911; Kosaka, 1912; Clark, 1926; Weinberg, 1928; Schneider, 1930; Corbin, 1940; Corbin and Harrison, 1940 etc.). As early as 1871 some authors had assumed that this nucleus might be sensory in nature (Meynert, 1871; Krause, 1876; Bechterew, 1899). But it was Johnston (1909) who clearly established this fact through extensive studies of the mesencephalic tract and nucleus in the vertebrate kingdom. The principal reason for his judgment as to the sensory character of this nucleus was the morphological features of the nerve cells, while its embryological origin from the dorsal, alar plate re-enforced this conclusion. Concerning the peculiarity that the peripheral nerve fibers continue directly to the sensory cells in the brain stem, the same situation was observed in the spinal ganglia of Amphioxus. Furthermore, such a thing is said to occur partially also in the embryos of the petromyzon, fishes, amphibians and reptiles. Therefore Johnston was led to the conclusion that the mesencephalic fifth nucleus represents a sensory ganglion which had originally derived from the neural crest and was taken in the brain stem.

The question, whether or not this mesencephalic nucleus receives afferent impulses only from the masticatory muscles, is still left open. Some authors claim that a part of the fibers from the mesencephalic tract are distributed peripherally through the portio major (Johnston, 1909; Kosaka, 1912; Thelander, 1924 and others). Corbin (1940) destroyed the mesencephalic tract and nucleus in many cats and examined degenerated fibers in the peripheral nerves. He found that in addition to nerves to the masticator muscles, the following nerves on the ipsilateral side showed degenerated fibers: ethmoidal branches of the ophthalmic nerve, palatine and superior alveolar branches of the maxillary nerve, inferior alveolar branches of the mandibular nerve, etc. Thus it was concluded that afferent impulses from the palate, gingiva and the periodontium would be useful for controlling the chewing force efficiently so as to protect the gingiva and palate from damage.

Although May and Horsley (1910) and Allen (1919) assumed that some fibers of the mesencephalic fifth tract derived from the cells in the semilunar ganglion and were connected to the oculomotor and trochlear nuclei, such an opinion has not been generally accepted. Also the nucleus loci caerulei had often been described in error as giving rise to some fibers of this tract.

The relationship between the mesencephalic tract or nucleus and striated muscles other than the masticator muscles in the trigeminal nerve territory is not yet very clear. Kölliker (1896), who regarded this nucleus as motor in nature, assumed that it might innervate the m. tensor veli palatini and m. tensor tympani. Terterjanz (1899) wrote that, after the removal of the cat's tensor veli palatini, some fibers of the mesencephalic tract underwent degeneration, whereas the same experiment of Kure (1899) in the dog presented negative results.

Concerning m. tensor tympani, m. mylohyoideus, and the anterior belly of the digastric muscle, also, no positive proof of their relationship to the mesencephalic tract or nucleus has been obtained (Kosaka, 1912; Corbin, 1940). Baum (1900) found no muscle spindles in either belly of the digastric muscle, while Gregor (1904) saw them in the mylohyoid.

Regarding the relationship between the mesencephalic tract or nucleus and the cranial nerves other than the trigeminus, ocular motor nerves were discussed in the preceding chapter.

There are some reports that the sectioning of the facial or hypoglossal nerve roots provoked tigrolysis in the mesencephalic nucleus (Lewy, Groff and Grant, 1938). It may need further corroboration.

The ascending pathway from the mesencephalic fifth nucleus is still questionable. If Cajal's illustration is right, the cells of this nucleus have no neurite. The collaterals extending to the masticatory nucleus (nucl. motorius n. trigemini) represent the only path for continuing impulses from the periphery. So these collaterals may be axons, which completes a closed reflex arc as follows: Masticatory muscles → mesencephalic nucleus → masticatory nucleus → masticatory muscles. It is however not improbable that fine neurites do exist, which are escaping our observations. Schneider (1928) described terminal rod like structures on the surface of cells of the dog's mesencephalic nucleus, which were stained with silver technique.

In passing, Sano (1959) examined many patients of the trigemino-oculomotor synkinesis, especially the pterygoid-levator synkinesis, where the opening of the mouth caused reflective elevation of the upper eye lid (Marcus Gunn's phenomenon), and assumed a reflex arc originating in the masticator muscles to be transmitted through the mesencephalic fifth tract to the oculomotor nucleus.

III. Sensory Innervation of the Facial Muscles

1. Are the facial muscles furnished with proprioceptive sensation?

In his book on "the examination of reflexes," Wartenberg (1945) discussed minutely the orbicularis oris reflex and emphasized that it is neither a skin-muscle reflex nor a periosteum-muscle reflex but it represents evidently a muscle-muscle reflex. So he postulated the existence of afferent discharges from the orbicularis oris muscle. But the morphological proof of this sensory innervation of facial muscles has not been demonstrated clearly and we have only vague knowledge about this problem.

Concerning sensory nerve terminations in the facial muscles, Hines (1927) cited that Cipollone (1897) found no muscle spindle in the rabbit and that Smith (1926) obtained the same negative result in the embryo of the pig, cat, guinea-pig and rat.

In human material, Kadanoff (1956) examined the striated muscles of the lip, nose, cheek and auricle with Bielschowsky's silver stain, and encountered a very few, but typical muscle spindles as well as some atypical spindles without connective tissue capsule. In addition he saw several types of epilemmal nerve endings such as the terminal loops, terminal boutons and the mixed type. Also terminal glomeruli were observed in the interstitial connective tissue.

According to him these afferent end-apparatuses were better developed in the muscles of the lip and oral angle than in the nose, cheek and auricle. Needless to say, the former represent more movable parts of the face. According to Okamoto's personal information, she found spiral nerve endings in the human orbicularis oris, which were quite similar to those described by her in the extrinsic eye muscles (1957).

2. Peripheral course of sensory nerve fibers from the facial muscles.

Naturally one may think at first of the trigeminal nerve as the peripheral course of afferent impulses from the facial muscles. As a matter of fact, however, this course had been neglected and the facial nerve itself was subjected to the attention of many authors, because many clinical and clinico-experimental cases were known, where the complete destruction or removal of the semilunar ganglion still left some deep sensibilities or pressure and pain sensations in the face (Spiller, 1906; Ivy and Johnson, 1909; Gerard, 1923; Davis, 1923 etc.).

Some authors attributed this to the incompleteness of operation or assumed that this sensation might be conducted by the autonomic nervous system (Mills, 1910; Maloney and Kennedy, 1911).

Huber (1930) did a self-experiment with the help of Hughson and, after complete anaesthesia of the trigeminal nerve with novocain, he could still judge accurately and promptly the site of muscle contractions caused by direct electrical stimulation of facial muscles. Thus Huber was led to the conclusion that the facial musculature is furnished with proper discriminative sensations, which are probably transmitted through the facial nerve trunk.

In the clinical experiments of Carmichael and Woollard

(1933), no pressure and pain sensations were recognized after the anaesthesia of the semilunar ganglion. The experimentees could, however, feel the swelling or bulging of the cheeks when they ate food, and this feeling was lost when the facial nerve was blocked, implying the existence of some proprioceptive sensations travelling through the facial nerve.

Early in this century Head (1905) wrote in his famous article, where he discriminated three sensory factors, the epicritic and protopathic sensation and the deep sensibility, that the deep sensibility, which feels the pressure and movement as well as pain by powerful pressure, is transmitted by afferent nerve fibers contained in the motor branches and is not impaired by cutting the cutaneous sensory nerves. Of course he was led to this rule through his observations in the spinal nerve territory. But, judging from above mentioned reports on the facial and trigeminal nerves, this rule may hold good to some extent also for the facial areas. It is however not easy to know the exact relationship between the so-called deep sensibility and the proprioception of muscles.

Morphological studies have shown that a few afferent fibers are present in the facial nerve trunk at the level of the stylomastoid foramen (Weigner, 1905 and others). Foley and DuBois (1943) analyzed the fiber composition of the cat's facial nerve and made it clear that of all the nerve fibers amounting to some 20,000 (18,910–28,471), motor and autonomic fibers occupied about 69% and 15% respectively, while the remaining 15% were represented by the sensory fibers. Regarding these sensory fibers, more than the half (45–62%) were distributed to the chorda tympani, while n. petrosus major took some one third of the total number (27–40%) and only a few fibers (11–15%), of which the real number was 109–442, were shown to accompany the main trunk peripherally.

According to the report of Wakeley and Edgeworth (1933) in the monkey, the facial nerve trunk had approximately 5,000 medullated nerve fibers just below the stylomastoid foramen and about 7% (359 fibers) were shown to be afferent.

Concerning the peripheral origins of these sensory fibers, there are roughly speaking two kinds of opinions. One of them regards these fibers exclusively in association with the posterior auricular branches which convey the pain sensation from the auricular

skin (Hunt, 1909, 1937; Rhinehart, 1918; Larsell and Felton, 1928; Foley and DuBois, 1943; Pearson, 1945-b; Brodal, 1947). The study of Hunt (1937) was based on the distribution of herpes zoster and the analysis of symptoms of geniculate neuralgia, and he published a schema to illustrate the peripheral courses of afferent fibers in the facial nerve (Fig. 10).

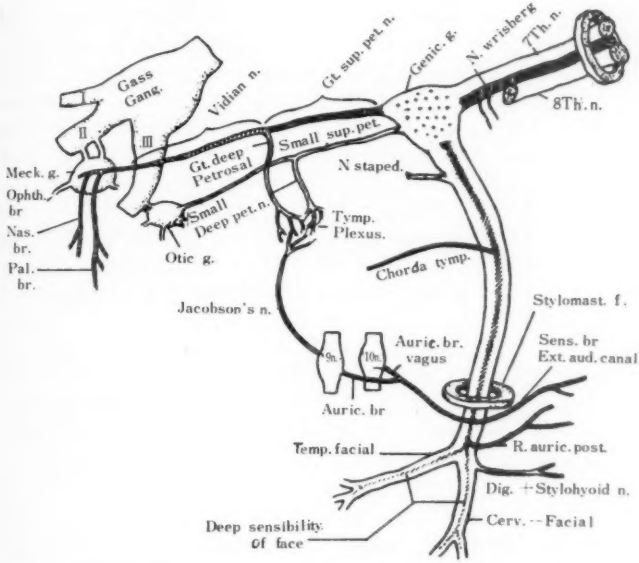


FIG. 10. Diagrammatic representation of the facial nerve, showing the geniculate ganglion and its sensory root and peripheral divisions. The sensory system is shaded. Hunt, 1937)

As is well known, the auricular skin is innervated also by the auricular ramus of the vagus nerve. As a matter of fact, the just mentioned ramus of the tenth nerve (Arnold's nerve) in the dog, cat, rat, etc. joins the facial nerve trunk temporarily and is distributed to the auricular area together with the posterior auricular branches.

The second viewpoint about the afferent fibers of the facial nerve accepts the possibility that these fibers are distributed widely in the facial areas so as to convey the deep sensibilities or proprioceptive impulses from the facial muscles. Clinical and

clinico-experimental reports mentioned in the beginning of this paragraph obviously support this second opinion.

Morphological proof of this viewpoint was proposed by Bruesch (1944) to some extent (Fig. 11). He repeated degenera-

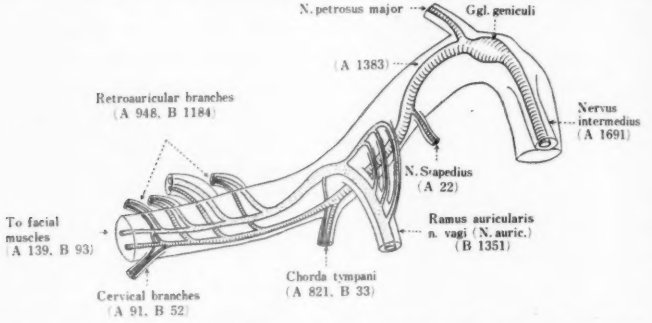


FIG. 11. Afferent medullated nerve fibers in the cat's facial nerve. (Bruesch, 1944) (A: average number of afferent fibers from the intermediate nerve. B: average number of afferent fibers from the auricular ramus of vagus nerve.)

tion experiments in more than twenty cats and made it clear that at least some 140 fibers from the facial trunk, together with some 90 afferent fibers deriving from Arnold's nerve, are distributed to the facial muscles. According to him the posterior auricular branches comprise on an average 948 afferent fibers from the facial and 1184 from the vagus nerve. Thus the number of sensory fibers distributed to facial musculature is relatively very scanty. Bruesch found no sensory end apparatus in the muscles, excepting some free endings in the adventitia of blood vessels.

3. *Nucleus for receiving the afferent impulses from facial muscles.*

It has been accepted that the above mentioned afferent fibers in the facial nerve trunk distal to the stylomastoid foramen have their perikaryons in the geniculate ganglion, where also are situated the cells for fibers to be distributed through the n. petrosus major and chorda tympani to the mucous membrane of the tongue and the palate (Davis, 1923; Bruesch, 1944).

Concerning the central processes extending from bipolar cells of the geniculate ganglion, Kimmel (1941) examined silver

stained brain preparations of the rabbit's embryos in various stages of development and discriminated three types of destinations as follows: (1) A few fibers appear to turn caudad to terminate in the nucleus of the spinal tract of the trigeminal nerve. (2) The most fibers turn caudad so as to form the rostral part of the fasciculus solitarius. (3) Some fibers continue medially to end in a small-celled nucleus in the region of the internal genu of the facial nerve and medial to the abducens nucleus.

Afferent fibers from the ninth and tenth nerve were observed to join the first and second courses just mentioned. Since the solitary tract and its nucleus are regarded as receiving gustatory and mucous membrane sensations, the spinal tract of the trigeminal nerve and its nucleus may represent the destination of afferent fibers from the facial peripheral areas. However, no electro-physiological evidence has been obtained so far.

Brodal (1947) reported that, in all four cases of the clinico-experimental severance of the spinal tract of the trigeminal nerve, anaesthesia of the auricular skin was observed to accompany the disappearance of sensibilities in the proper trigeminal nerve regions. Taking into consideration the fact that the auricular region is innervated by the seventh, ninth and tenth nerves, Brodal concluded that the nucleus of the spinal tract is responsible for the sensory innervation of the auricular skin.

The connection between the afferent fibers of the intermediate nerve and the mesencephalic fifth tract was suggested by Pearson (1945-b), who observed in the brain of human fetuses that some fibers of the former nerve continue craniad beyond the fasciculus solitarius into the mesencephalic tract.

Some authors suppose theoretically that the superior sensory nucleus of the trigeminal nerve may receive the proprioceptive impulses from the facial muscles (Elliott, 1947). This hypothesis is based on the assumption that the nucleus of the spinal tract of the fifth nerve corresponds to the dorsal column of spinal cord, while the superior sensory nucleus is equivalent to the nuclei cuneatus and gracilis. It is at least accepted by Gerard (1923), Windle (1926) and Smyth (1939) that the nucleus of the spinal fifth tract is concerned with temperature and pain sensations of the face. The tactile sensation of the face is said to be transmitted not only to the superior sensory nucleus but also to the nucleus of the spinal tract.

The secondary, ascending pathway to convey facial proprioception is quite unknown. If such a tract exists, it may be mixed in the trigeminal lemniscus. This lemniscus is further divided into ventral and dorsal parts; for details the reader is referred to Wallenberg (1905), Walker (1939), and Smyth (1939).

In addition to the facial muscles, there are some more striated muscles in the territory of the seventh nerve: posterior belly of the digastricus, the stylohyoideus and the stapedius muscle. We have almost no knowledge about their afferent innervation of the former two. Baum (1900) saw no muscle spindles in them, whereas Gregor (1904) encountered some in the stylohyoid. The stapedius muscle will be discussed in the next chapter.

IV. Sensory Innervation of Muscles of the Auditory Ossicles

Two tiny striated muscles, the tensor tympani and the stapedius, attach to auditory ossicles and may serve to protect the inner ear by preventing the ossicles from too severe vibrations. They may also serve to make the mechanism more responsive under attention. It is well known that the stapedius receives a small branch (n. stapedius) from the seventh nerve trunk, while the tensor tympani is innervated by the n. mandibularis. But the anatomical and physiological details concerning proprioception of these muscles are scarcely known.

One may suppose that the auditory sensation will play the role of afferent arm of the reflex arc for controlling the movements of these muscles, leaving no necessity for sensory innervation of them. But the existence of receptor nerve endings in those muscles contradict this presumption.

Nerve endings in the muscles of the auditory ossicles were studied by Krebs (1905) and Ota (1958). The former author examined the ox's stapedius with methylene blue staining as well as with the gold chloride method, whereas the latter studied the cat's stapedius and tensor tympani by means of gold chloride method and Bielschowsky's silver stain. Concerning sensory endings, Krebs described one typical tendon spindle and some Ruffini body-like end apparatus. Tree-like terminations also were observed in the interstitium. A network of fine fibrils, probably of autonomic character, was seen in the walls of arterioles.

Ota found only one typical muscle spindle in twenty-five tensor tympani muscles, while a few spiral nerve endings were

noticed in the stapedius muscles. Besides, he discriminated in both muscles grape-like, bush-like, tree-like and rod-like endings. Ota studied further the relationship between the motor endings and muscle fibers, and described some such cases where a single muscle fiber was furnished with two end plates, of which each nerve fiber was directed opposite to the other.

Accepting that the muscles in question are supplied with sensory innervation, it is probable that the tensor tympani receives it from the same pathway and origin as that for the masticatory muscles. As referred to in the chapter II, however, nobody has obtained positive evidence for the connection between the tensor tympani muscle and the nucleus of the mesencephalic fifth tract (Kosaka, 1912; Corbin, 1940 etc.).

As regards the course and origin of afferent fibers from the stapedius muscle, one may easily think of their community with that of the facial muscles. But the delicate anatomical situation of this muscle has prevented the fruitful success of the study.

According to the study of Foley and DuBois (1943) in the cat, n. stapedius is mainly composed of medullated fibers 2.5–4.5 μ in diameter, of which the number was about one thousand, occupying some 10% of the total somatic efferent fibers of the seventh nerve. They encountered a few nerve cells in the stapedius nerve and regarded them as heterotopic elements of the autonomic nature.

Bruesch (1944) showed by degeneration experiments in the cat that a few medullated nerve fibers (on an average 22 fibers) arising in the geniculate ganglion were distributed to the stapedius nerve (Fig. 11).

V. Sensory Innervation of the Pharyngeal, Laryngeal and Palatine Muscles

Compared with the extraocular, masticatory, facial muscles etc., those striated muscles of the pharynx, larynx and palatum, innervated by the ninth and tenth nerves, show a tendency towards somewhat involuntary or reflex character. They represent so to say the intermediate type between the "somatic motor" and "visceral motor" muscles. They are therefore called sometimes "special visceral motor" muscles, whereas the general visceral, smooth muscles are called "general visceral motor." They may

be better called "branchial" muscles. Nucl. ambiguus is said to innervate the musculature in question.

Before discussing the proprioception of these muscles, general afferent fibers comprised in the glossopharyngeal and vagus nerves will be recalled. As is well known, one group of sensory fibers convey cutaneous sensations of the auricle through the ramus auricularis nervi vagi. The proper ganglion calls are located mostly in the superior or jugular ganglion (DuBois and Foley, 1937), and their central processes, as presented in chapter III, are said to reach the nucleus of spinal tract of the trigeminal nerve (Kimmel, 1941; Brodal, 1947).

The second group of afferent fibers concern the general sensation of mucous membranes of the pharynx, larynx and palate, including gustatory sensation from the posterior part of the tongue. Nerve cells are considered to be in the inferior or nodosal ganglion of the tenth nerve as well as in both the superior and inferior ganglia of the ninteh nerve, of which the centripetal fibers probably continue to the nucleus solitarius (Foley & DuBois, 1934; Tarlov, 1940; Kimmel, 1941). Then the secondary tract ascends to the thalamus and further to the sensory area of the cerebral cortex (Allen, 1923-a,b).

Anatomical and physiological knowledge about the proprioceptive innervation of those muscles is very vague. Sensory nerve endings have been reported in them from time to time, although they appear to be poorly developed. A few muscle spindles were seen by Gregor (1904) in the stylopharyngeal muscle of the human fetus, by Slawik (1942) in the upper part of the human oesophagus, by Nakayama (1911) and Nakamura (1915) in the human laryngeal muscles, by Sugano (1929) in the dog's larynx, by Brocklehurst and Edgeworth (1940) in m. cricothyreoideus of the monkey, by Goerttler (1950) in the human vocal muscle, by by Winckler (1956) in the human lateral cricoarytenoideus, by Paulsen (1958-a,b) in the m. vocalis, cricoarytenoideus posterior and cricothyreoideus of man (Fig. 12).

Besides, apparently sensory nerve terminations were described by Sunder-Plassmann (1933-a,b) and Mündich (1938) in both the m. vocalis and cricoarytenoideus of man and rabbit respectively. Thus it is clear that those muscles are supplied, although scantily, with afferent innervation.

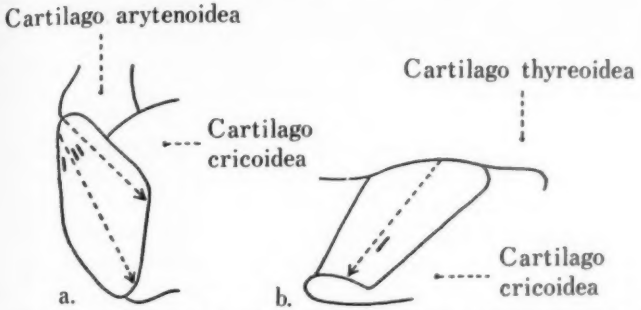


FIG. 12. Distribution of muscle spindles in the human laryngeal muscles. (Paulsen, 1958) (a: M. cricoarytenoideus posterior, b: M. cricothyreoideus.)

Nakamura (1915), who found muscle spindles in the laryngeal muscles of the patients having infantile beriberi, doubted the opinion of Kiyono (1911) that, in the beriberi patients, intrafusal fibers of muscle spindles were subjected to higher pathological changes than the ordinary muscle fibers.

When Strong (1945) observed that some fibers of the m. vocalis went into the vocal ligament so as to end spirally, he supposed peculiarly that these muscle fibers might serve to feel the tension of the vocal ligament. Motor nerve endings in the laryngeal muscles were studied by Grabower (1902), Arione (1923), Takasaki (1928) and others.

Although the exact courses of afferent fibers from the laryngeal, pharyngeal as well as palatine muscle are not known, some reports will be mentioned here which may give clues for future researches.

Judging from his degeneration experiments in the monkey and cat, Sherrington (1894) stated that the laryngeal recurrent nerve contains some sensory fibers deriving from the cervical spinal ganglia.

Foley and DuBois (1937) sectioned the trunk of cat's vagus nerve and traced some intact fibers of small diameter (2–3 μ) into the recurrent nerve and further probably into the tracheal and oesophageal branches. These authors could not, however, ascertain whether or not some of these intact fibers reach the laryngeal muscles. According to them, the vagus trunk just below the inferior ganglion did not include many medullated fibers

(23–33%), of which the majority (54–64%) were motor, the rest (36–46%) representing probably the sensory fibers. On the average some 25,000 ganglion cells were counted in the inferior ganglion, some of which showed chromatolysis after the section of the recurrent nerve.

Brocklehurst and Edgeworth (1940), after sectioning monkey's vagus trunk, found that some 3% of fibers in the recurrent nerve, just before entering the laryngeal muscles, were not degenerated and regarded them as proprioceptive fibers. On the other hand, some 30% of fibers of the superior laryngeal nerve were intact, which may serve for transmitting the sensory impulses from mucous membranes etc.

Fiber-analytical studies of laryngeal nerves, especially of the inferior laryngeal nerve, were done by Tomasch and Britton (1955), Murray (1957), Faaborg-Andersen (1957) etc., and it was shown that this nerve presents a distribution curve with one peak at the site a little less than 10 μ of the diameter. Since it is said that nerves innervating muscles with many muscle spindles usually contain a considerable number of thin fibers and present a distribution curve with two peaks, the above observation may suggest that the laryngeal muscles are poorly supplied with proprioceptive innervation (Murray, 1957). According to Paulsen (1958-a,b), only one muscle spindle was found in four human vocal muscles, one spindle each in two muscles of four cricothyreoideus, and one to five spindles in each of five posterior cricoarytenoid muscles.

Hirose (1960, personal information) severed the recurrent nerve of cat and recorded action potentials at the proximal end of the peripheral stump, when he exerted a slight pressure on the larynx. However, it could not be decided whether these impulses derived from muscles or from mucous membranes.

VI. Sensory Innervation of the Sternocleidomastoideus and Trapezius Muscles

The sternocleidomastoideus and trapezius, innervated by the accessory nerve, are surely equipped with proprioceptive nerve supply. Especially in the former muscle, a considerable number of muscle spindles were found (Baum, 1900; Gregor, 1904; Willard and Lawrence, 1933; Voss, 1958) and reflex movements apparently of the muscular origin were observed (Sano, 1953).

Concerning the quantitative relation of muscle spindles in muscles in general, various methods of measurement have been proposed: (1) largest number of spindles in the cross-section of muscles (Gregor, 1904), (2) number of spindles in an unit area of the cross-section (Oshima, 1938), (3) density of spindles, which is given by the average number of spindles per one gram of the muscle, that is to say, the ratio between the total number of spindles and the weight of muscle in grams (Oshima, 1938; Voss, 1937, 1958; Schulze, 1955; Kiesewetter, 1959; Körner, 1960) (see Table 2).

TABLE 2

Distribution density of muscle spindles in different muscles. (The density is given by the average number of spindles per one gram of the muscle)

Man, lumbrical muscles of hand (Voss, 1937)

	Weight of muscle (g.)	Total number of spindles	Density of spindles
I. lumbr. m.	3.1	51	16.5
II. lumbr. m.	1.8	36	19.7
III. lumbr. m.	1.6	20	12.2
IV. lumbr. m.	1.3	23	17.5
Average			16.4

Man, lumbrical muscles of foot (Voss, 1937)

	Weight of muscle (g.)	Total number of spindles	Density of spindles
I. lumbr. m.	1.7	36	21.0
II. lumbr. m.	1.5	26	17.5
III. lumbr. m.	1.4	17	12.1
IV. lumbr. m.	1.2	24	19.5
Average			17.5

Chimpanzee, lumbrical muscles of hand (Voss, 1937)

	Weight of muscle (g.)	Total number of spindles	Density of spindles
I. lumbr. m.	0.37	46	12.5 (125)
II. lumbr. m.	0.60	31	5.2 (52)
III. lumbr. m.	0.48	25	5.2 (52)
Average			7.6 (76)

TABLE 2—Continued

Distribution density of muscle spindles in different muscles. (The density is given by the average number of spindles per one gram of the muscle)

Chimpanzee, lumbrical muscles of foot (Voss, 1937)

	Weight of muscle (g.)	Total number of spindles	Density of spindles
I. lumbr. m.	0.37	36	9.7 (97)
II. lumbr. m.	0.33	27	8.1 (81)
III. lumbr. m.	0.43	24	5.5 (55)
Average			7.7 (77)

Cat's muscles (Oshima, 1938)

	Weight of muscle (g.)	Total number of spindles	Density of spindles
III. lumbrical muscle of fore limb (average of 4)	0.04	7	17.3 (173)
M. sartorius (2)	11.4	66	6.1
II. external intercostal m. (3)	0.7	18	2.7 (27)
M. extensor digit. comm. (2)	1.3	22	1.8 (18)
M. flexor digit. superf. (2)	2.2	35	1.7 (17)
M. extensor carpi rad. long. (4)	1.3	18	1.4 (14)
M. flexor carpi radialis (4)	1.3	18	1.4 (14)
M. biceps brachii (4)	6.4	53	0.9 (9)
M. rectus abd. (2)	20.46	87	0.4 (4)

Kitten's muscles (Oshima, 1938)

	Weight of muscle (g.)	Total number of spindles	Density of spindles
III. lumbrical muscle of fore lumb (1)	0.004	7	175 (1750)
M. flex. carpi rad. (1)	0.13	17	13.1 (131)
M. ext. carpi rad. long. (1)	0.12	15	12.5 (125)
II. external intercostal m. (1)	0.1	21	21.0 (210)
M. biceps brachii (1)	0.5	45	9.0 (90)
M. rectus abd. (1)	19.0	85	4.5

TABLE 2—Continued

Distribution density of muscle spindles in different muscles. (The density is given by the average number of spindles per one gram of the muscle)

Man, thenar muscles (Schulze, 1955)

	Weight of muscle (g.)	Total number of spindles	Density of spindles
			11.6–29.2

Man, muscles of pectoral girdle (Kiesewetter, 1958)

	Weight of muscle (g.)	Total number of spindles	Density of spindles
M. levator scapulae	41.1	94	2.29
Mm. rhomboidei	93.6	119	1.27
M. teres minor	27.7	35	1.26
M. teres major	122.7	44	0.36
M. supraspinatus	51.8	28	0.54
M. infraspinatus	137.1	54	0.39
M. infrascapularis	182.8	83	0.45
M. deltoideus	355.7	182	0.51
M. serratus anterior	197.5	171	0.87

Man, infrahyoid muscles (Voss, 1958)

	Weight of muscle (g.)	Total number of spindles	Density of spindles
M. sternohyoideus	6.1	41	6.5
M. sternothyreoideus	6.2	86	13.8
M. thyreohyoideus	2.0	12	6.0
M. omohyoideus	6.2	83	13.3
Superior belly	...	51	...
Inferior belly	...	32	...
M. sterno-cleidomastoideus	53.4	303	5.6

Man, abdominal muscles (Voss, 1958)

	Weight of muscle (g.)	Total number of spindles	Density of spindles
M. rectus abdominis	157.3	354	2.25
M. obliquus ext. abd.	161.2	569 (590)	3.50 (3.6)
M. obliquus int. abd.	109.2	332	3.04
M. transversus adbdominis	58.2	425	7.30

TABLE 2—Continued

Distribution density of muscle spindles in different muscles. (The density is given by the average number of spindles per one gram of the muscle)

Man, Musculi nuchae profundi (Voss, 1958)

	Weight of muscle (g.)	Total number of spindles	Density of spindles
M. rectus capitis posterior major	4.0	122	30.5
M. rectus capitis posterior minor	1.5	54	36.0
M. obliquus capitis superior	3.3	141	42.7
M. obliquus capitis inferior	7.9	232	29.3

Man, muscles of shoulder joint (Körner, 1960)

	Weight of muscle (g.)	Total number of spindles	Density of spindles
M. pectoralis major	295.6	450	1.52
M. pectoralis minor	50.5	87	1.72
M. sternalis	...	2	...
M. subclavius	5.2	27	5.19
M. coracobrachialis	39.8	123	3.09
M. biceps brachii	163.8	320	1.95
M. brachialis	141.0	256	1.81
M. triceps brachii	364.4	520	1.42
Caput longum	138.4	222	1.6
Caput laterale	94.2	200	2.12
Caput mediale	92.5	98	1.06

In addition, the ratio in number between the ordinary muscle fibers and spindles (in the sternocleidomastoideus 10,895:1, Gregor, 1904) as well as between the motor units and muscle spindles were counted (in the cat's tibialis anterior nearly 2:1, Hagbarth and Wohlfahrt, 1952).

The data obtained so far do not cover the whole musculature and it is still impossible to draw conclusions about the distribution of muscle spindles in the body of man and animals. Generally speaking, however, it is probable that, in the extremities, the more peripheral muscles are furnished with more numerous spindles. Muscles capable of delicate degrees of movement may need many spindles.

It is said that muscles of the upper extremity usually have more spindles than the equivalent muscles of the lower extremity and that the muscles of the trunk possess fewer spindles than those of extremities. Cervical muscles are also regarded as relatively rich in muscle spindles.

The accessory nerve, which controls the sternocleidomastoideus and trapezius, is composed of cranial and spinal roots, of which the former is merely the accessory root of the vagus nerve. Only the spinal roots are responsible for the innervation of those muscles. In addition, ventral rami of some of the C3 and C4 contribute anastomoses to the accessory nerve.

With regard to the conducting pathway of proprioceptive impulses from the accessory musculature, three possibilities have been proposed:

(1) The accessory nerve trunk itself can be the route in question, for some of the root fibers were traced into the spinal cord toward the dorsal column as well as cranially near to the inferior end of the solitary tract (Darkschewitsch, 1885; Windle, 1931-b; Pearson, 1937, 1938). Needless to say, the majority of fibers, motors in nature, were directed ventrally to the nucl. originis n. accessorii.

(2) The second theory accepts the anastomoses between the spinal root of accessory nerve and the dorsal roots of upper cervical nerves (C1, C2) inside the vertebral canal, and assumes that some of the originally spinal afferent fibers travel through these anastomoses into the accessory nerve trunk.

Although Holl (1878) and Kazzander (1891) did not notice such anastomoses and thought that the accessory nerve root and cervical dorsal roots were independent of each other, Weigner (1901) described various types of communications between the two and, according to him, sometimes the dorsal root of C1 appeared to be the root of the accessory nerve itself.

On the other hand, clusters of nerve cells have been reported in various sites of the accessory nerve root, both inside and outside the spinal or cranial cavity (Streeter, 1905; Fahmy, 1927; Hakamada, 1943; Pearson and O'Neil, 1946) (Fig. 13). According to Windle (1931-b), the average number of ganglion cells on each side was 120 (35–235) in the monkey and 44 (12–96) in the cat. Some of the cells were surrounded by mantle-cells.

Furthermore, after sectioning the accessory nerve just before

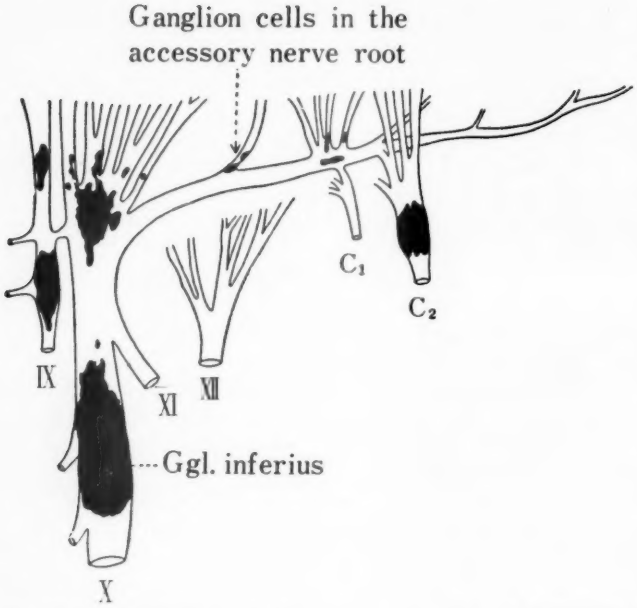


FIG. 13. Nerve cell clusters in the accessory nerve roots of human adult. (Streeter, 1905)

entering the sternocleidomastoideus, Windle found chromatolysis in those cells as well as in a considerable number of cells in the spinal ganglion of C1. He concluded that these cells in the accessory nerve trunk belonged to the same category as those of the spinal ganglia.

However, when Windle and Lozier (1932) severed the eleventh nerve of cat and stimulated the central stump, no sign of afferent responses such as observed by stimulation of general sensory nerves was noticed and the authors were led to conclusion that the nerve cells in the accessory trunk must convey exclusively proprioceptive impulses from the muscles. Windle accepted the co-existence of both the first and second pathways (Fig. 14).

(3) The third viewpoint denies the anastomoses between accessory and cervical nerve roots inside the vertebral canal. The

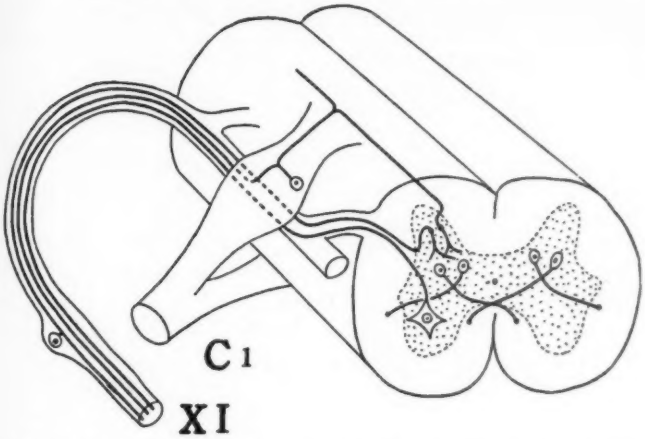


FIG. 14. Pathway of afferent neurons in the accessory nerve. (Windle, 1931)

eleventh nerve itself is regarded as purely motor in nature, at least in its proximal part around the jugular foramen. Anastomoses occur only in the more peripheral part and some sensory fibers deriving from ventral rami of the upper cervical nerves are considered to join the accessory nerve and through them reach the target muscles. There may be also some sensory fibers which innervate the muscles directly from cervical nerves.

This viewpoint was advocated by Corbin and his school. At first Hinsey and Corbin (1934) threw doubt upon the second theory, when, after removing the spinal ganglion of C1 of the cat, they found no degenerated fibers in the accessory nerve trunk at the middle between the jugular foramen and the sternocleidomastoideus.

Subsequently Corbin, Lhamon and Petit (1937) got the same negative result after removing the spinal ganglion of C2 of the monkey, while they saw a few degenerated fibers in the hypoglossal nerve. Thus they intended to look for a new pathway for transmitting afferent impulses from the accessory musculature by means of degeneration experiments and the electro-physiological method (Corbin and Harrison, 1938, in cats; Yee, Harrison and Corbin, 1939, in rabbits). They were not able to record action potentials, by stretching the sternocleidomastoideus, in

the accessory nerve trunk just below the jugular foramen, while they noticed clear responses in the ventral rami of the upper cervical nerves. The degeneration experiments also testified to, and elaborated on, this conclusion (Fig. 15).

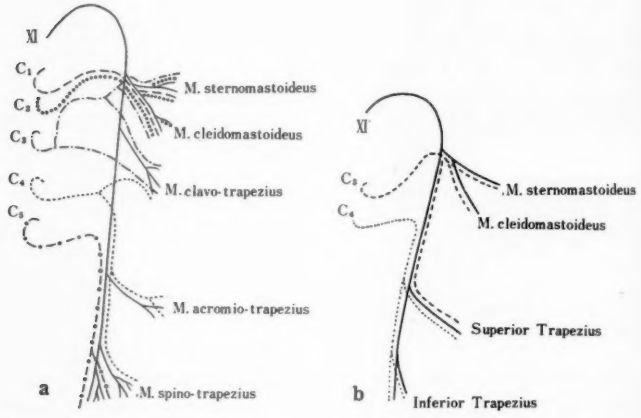


FIG. 15. Sensory fibers from upper cervical nerves innervating the accessory musculature.
a: Cat. (Corbin & Harrison, 1938)
b: Rabbit. (Yee, Harrison & Corbin, 1939)

Nothing can be said as yet about the nucleus for receiving proprioceptive impulses from the sternocleidomastoideus and trapezius. However it is probably noteworthy that many authors mentioned the close relationship between the ascending fibers from dorsal roots of upper cervical nerves and the nucleus solitarius (Windle, 1931-a; Ranson, Davenport and Doles, 1932; Corbin and Hinsey, 1935; Corbin, Lhamon and Petit, 1937; Yee and Corbin, 1939).

According to them, some fibers from the upper cervical dorsal roots ascend medialward in the cuneate fasciculus, turn rostrally and continue to the inferior end of the solitary tract, so as to end in its nucleus. Though interesting, it is not an easy problem to decide whether or not the nucleus solitarius is concerned with the proprioception of the accessory musculature.

VII. Sensory Innervation of the Lingual Muscles

1. Sensory nerve endings in the lingual muscles

Although many papers have been published on the proprioceptive innervation of lingual muscles, a kind of uncertainty still prevails, just as in the case of other muscles discussed in the foregoing chapters. Even with regard to the sensory nerve endings, some authors have described muscle spindles, whereas others failed to find them in spite of their careful investigations. There must be a considerable variability among different animals and individuals. One may wonder what kind of physiological significance is to be attributed to such variable or labile structures.

The following authors did not see muscle spindles in the lingual muscles: Sherrington (1894, cat, monkey), Batten (1897, man), Cipolonne (1897, man), Baum (1900, man), Boeke (1927, mouse, rat, echinus), Hewer (1935, man), Carleton (1938, rabbit, chameleon, ant-eater), Yee, Harrison and Corbin (1939, cat, rabbit, monkey, rat, man, guinea-pig, opossum), Cooper (1953, cat) and others. Weddell, Harpman, Lambley and Young (1940, rabbit, rat), as well as Boyd (1941, rabbit), also found no muscle spindles, but they encountered some atypical, probably sensory nerve terminations in some of the extrinsic lingual muscles (m. genioglossus etc.).

Authors who had the opportunity to see muscle spindles in the tongue musculature are as follows: Franqué (1890, man), Pilliet (1890, man), Forster (1894, man), Gregor (1904, human styloglossus), Kiyono (1911, human intrinsic lingual muscle), Langworthy (1924, cat, dog, pig, opossum, rat), Hatano (1925, man, dog), Stöhr (1928, man), Schaffer (1933, man), Tarkhan (1936, rabbit), Nakayama (1944, man), Cooper (1953, man, monkey, genioglossus of a young goat), and others.

According to Nakayama (1944), who examined the lingual muscles of the human fetus with Bielschowsky's silver stain, spiral endings of the muscle spindle appeared first in the tenth fetal month. He saw them with the frequency of one spindle in every two preparations, of which the size was 12×1.5 cm. Nerve fibers concerned were several times as thick as the motor fibers.

Muscle spindles in the hyoid muscles, especially of the suprahyoidal group, were partly referred to in the sections II and III. Among the infrahyoid muscles, m. sternothyreoideus (Baum,

1900 etc.) and omohyoideus (Forster, 1894) are known to be equipped with spindles. Also in the sternohyoideus and thyreohyoideus, muscle spindles were found and a detailed numerical investigation was made (Table 2).

2. Sensory pathway from the lingual muscles

Three routes seem reasonable as the peripheral course of proprioceptive impulses from the lingual muscles.

(1) The hypoglossal nerve itself may mediate the afferent impulses. This possibility was proposed by Langworthy (1924-a, b), who stated that muscle spindles found in the tongue musculature disappeared by sectioning the hypoglossal nerve root. Then Tarkhan (1936-a, b) cut the same nerve in the rabbit and found afterward a few undegenerated nerve fibers in its intramuscular branches. At the same time he found nerve cells scattered around the site of branching of the ramus descendens and regarded them in association with the above mentioned undegenerated fibers, attributing to them the mediation of proprioceptive impulses.

Although Carleton (1938) tried the same experiment in the rabbit and failed to find undegenerated fibers, Tarkhan and Abou-El-Naga I (1947) extended their study to dogs and confirmed Tarkhan's earlier work. At the same time they noticed that removal of the inferior (nodosal) ganglion of the vagus nerve provoked degeneration in some peripheral fibers of the twelfth nerve, implying quite a new conception of the pathway of afferent fibers contained in this nerve trunk. According to them, removal of the superior (jugular) ganglion did not show any degenerative change at all in the hypoglossal nerve.

Some authors observed that stimulation of the central stump of the cut hypoglossus nerve produced various reflex responses like changes in the pupillary size, blood pressure or the nictating movements, and interpreted this phenomenon as proving the existence of afferent fibers in this nerve (Acheson, Rosenblueth & Partington, 1936: Downman, 1939).

Ganglion cells described by Tarkhan (1936) in the rabbit's hypoglossal nerve had been mentioned previously by Langworthy (1924-a, b), who, however, thought they were too scanty to take responsibility of proprioceptive innervation of lingual muscles. While Boyd (1937, 1941), Carleton (1938), Yee, Har-

rison and Corbin (1939), and Weddell *et al.* (1940) failed to find such nerve cells in the rabbit's hypoglossal nerve, Pearson (1943-b, 1945-a) noticed bipolar cells among the hypoglossal nerve root fibers inside the brain stem of a human embryo, and Tarkhan and Abd-El-Malek (1950), after the careful examination of the whole extent of hypoglossal nerves of rat, rabbit, cat, dog, and man, found clusters of several to some eighty nerve cells in nearly half of the examined cases (in 28 among 74 sides). Occasionally even a ganglion of more than one hundred cells was encountered.

These cell clusters found in the hypoglossal nerve trunk naturally remind us of the Froriep's ganglion or ganglion hypoglossi. Originally the so-called hypoblossal nerve is represented by the ventral root of the occipital spinal nerve, the dorsal root of which, although usually disappearing completely, sometimes is preserved in the pig, ox, deer, dog, bear, musk-cat etc. as the dorsal hypoglossal root. When the ganglion is preserved also on this root, it represents the Froriep's ganglion (Froriep & Beck, 1895; Beck, 1895). Naturally the ganglion of Froriep lies in the close vicinity of the brain stem, while the nerve cells described by Tarkhan *et al.* are distributed along the peripheral course of the hypoglossal nerve.

(2) Barron (1936), through his electro-physiological studies in the cat and rabbit, was led to the conclusion that the proprioceptive impulses from the tongue musculature are conducted by the lingual nerve, a branch of n. mandibularis of the trigeminal nerve. He never succeeded in recording action potentials in the hypoglossus nerve trunk, even when any kind of stimulation was added to the tongue. On the contrary, electro-physiological responses were obtained in the lingual nerve by stretching the tongue. Similar responses were recorded also in the chorda tympani as well as in the n. intermedius only when the tongue was stimulated chemically with M/20 solution of HCl, M/40 solution of NaOH, NaCl etc.

Barron supposed that the nerve cells concerned with lingual proprioception would be located in the semilunar ganglion or in the nucleus of the mesencephalic fifth tract. It is possible, however, that the afferent impulses Barron recorded were derived not from the muscles but from mucous membranes of the tongue.

Maloney and Kennedy (1911) reported clinical cases, where

the removal of semilunar ganglion still left some deep sensibilities in the face and tongue, which they explained by saying sensory fibers travel via the sympathetic nerves.

According to Weddell, Harpman, Lambley and Young (1940), who examined the difference in sensation mediated by the lingual nerve and inferior alveolar nerve, the complete anesthesia of both nerves did not cause any sign of ataxia of the tongue. It was also shown by degeneration experiments in rat and rabbit that some autonomic fibers from the superior cervical ganglion run in the twelfth nerve so as to innervate the blood vessels of the tongue.

(3) Authors who support the third viewpoint attribute the role of mediating lingual proprioception to the sensory fibers derived from the upper cervical nerves. In the last century, Sherrington (1894) showed, by removing the spinal ganglia of the monkey and cat, that the hypoglossal nerve contained some afferent fibers from the spinal ganglia.

Afterwards Van der Sprenkel (1924) traced peripherally the fibers from the echidna's spinal ganglion of C2 and found that some were distributed through the descending ramus of XII to the thyreoglossus, hyoglossus, styloglossus etc.

As regards the serial works by Corbin *et al*, it was at first reported that, after removing the ganglion of C1–C4, no degenerated fibers were encountered in the cat's hypoglossal nerve (Hinsey & Corbin, 1934). Although a few fibers (1–2%) underwent degeneration in the similar experiment on the monkey (Corbin, Lhamon & Petit, 1937), electro-physiological study in cats detected afferent responses by stretching the tongue neither in the hypoglossal trunk, n. lingualis nor in the cervical nerve roots (C1–C3) (Corbin & Harrison, 1938).

Thus they were obliged to conclude that the proprioceptive innervation of the tongue musculature peculiarly had nothing to do with any of the peripheral nerves, although they did not deny the possibility that the fibers in question might be too thin or scarce to be found by the ordinary degeneration method or the routine electro-physiological technique.

On the other hand Boyd (1937, 1941) studied the rabbit's lingual muscles as well as hypoglossus nerve and, considering also the relevant literature carefully, concluded that the upper

cervical nerves should represent the only possible way for transmitting the proprioceptive impulses from the tongue.

As if encouraged by this statement, Yee, Harrison and Corbin (1939) did experiments on the rabbit and repeated the observation of Corbin, Lhamon and Petit in the monkey (1937), corroborating the existence of a few degenerated, medium-sized fibers (1–5%) in the peripheral course of the twelfth nerve after removing the spinal ganglion of C1. Thus it seems to be fairly probable that at least some sensory fibers from the upper cervical nerves are participating in the afferent innervation of the tongue musculature (Fig. 16).

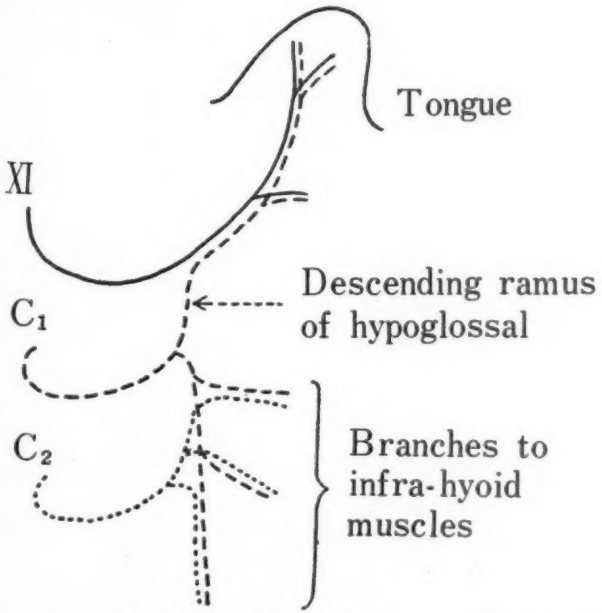


FIG. 16. Sensory fibers from the first cervical nerve to join the hypoglossus nerve. Rabbit. (Yee, Harrison & Corbin, 1939)

Concerning the fact that the tongue muscles receive afferent innervation from a higher cervical nerve (C1) than the accessory musculature (C2–C3), Yee, Harrison and Corbin explained that,

since the hypoglossus and accessorius nerve originally correspond to precervical and cervical segments respectively, it is rather natural that the hypoglossus co-operates with the higher cervical nerve than the accessorius.

Nothing definite is known about the secondary pathway in the brain stem for the proprioceptive impulses from the tongue muscles. Ascending tracts from the upper cervical nerves were mentioned in the last section.

In Conclusion

After reviewing the problem of proprioceptive innervation of muscles in the territory of cranial nerves, the author cannot but renew his wonder how so many questions and uncertainties regarding every detail of structure and function of human as well as animal bodies still exist.

Such factors as those mentioned in the preface have without doubt retarded progress of anatomical and physiological knowledge in this field. At the same time, something like the following should be admitted. Namely, authors who for instance have much knowledge about the morphology of nerve endings are generally not so good in the technique or interpretation of degeneration or physiological experiments, whereas those who are experts in physiological experiments are sometimes apt to be a little careless about the accurate topographical descriptions etc. Thus the integrative conclusion obtained cannot always avoid some uncertainties or obscureness.

If the authors of various specialties could work in really close co-operation with each other, it should not be difficult to arrive at more definite conclusions, even if limited to the techniques and methods which we already have. Needless to say, new methods and techniques should be devised and employed also. If, for instance, some electron-microscopical or histochemical method is found to distinguish the motor, sensory and autonomic nerve endings and cells, it will present a big help in solving the problem. Before long it might become possible to discriminate accurately various kinds of afferent impulses by the electro-physiological method.

Acknowledgments

This article was originally written in Japanese and printed in

the "Shinkei Kenkyû No Shinpo" (Recent Advances in Neurological Research) Volume 4 / No. 4, p. 827–860, Aug. 1960. At the suggestion of Dr. J. G. Sinclair of the University of Texas, this paper was, with pertinent revisions, translated into English. The author is indebted to him for kindly editing the English text. Acknowledgment is also due to Dr. T. Ogawa of the University of Tokyo for his thorough inspection and suggestions for the original manuscript.

After this translated paper went to press the author has seen the following analysis of proprioception in cranial nerves: Cooper, S. (1960): Muscle spindles and other muscle receptors. G. H. Bourne's "The structure and function of muscle. I. Chapter XI, pp. 381–420. Academic Press, New York and London.

REFERENCES

1. Abd-El-Malek, S.: On the presence of sensory fibres in the ocular nerves. *J. Anat.*, 72: 524–530, 1938.
2. Acheson, G. H., A. Rosenblueth, and Partington: Some afferent nerves producing reflex responses of the nictating membrane. *Amer. J. Physiol.*, 15: 308–316, 1936.
3. Allen, W. F.: Application of the Marchi method to the study of the radix mesencephalica trigemini in the guinea-pig. *J. Comp. Neur.*, 30: 169–216, 1919.
4. Allen, W. F.: Origin and distribution of the tractus solitarius in the guinea pig. *J. Comp. Neur.*, 35: 171–204, 1923-a.
5. Allen, W. F.: Origin and distribution of the secondary visceral fibers in the guinea pig. *J. Comp. Neur.*, 35: 275–311, 1923-b.
6. Allen, W. F.: Function of the cells in the motor root of the nervus trigeminus in the cat. *J. Comp. Neur.*, 38: 349–368, 1925.
7. Arione, L.: Beitrag zur Kenntnis der motorischen Endplatten der Kehlkopfmuskeln. (vorläufige Mitteil.) *Zb. f. Hals-Nasen u. Ohrenheilk.*, 3: 189–190, 1923.
8. Asuma, T.: On the innervation of the ventral abdominal wall in 10th month human embryo. *Tohoku J. Exp. Med.*, 55. (cited from Seto, '57) 1951.
9. Barker, D.: The innervation of the muscle spindle. *Quart. J. Micr. Sci.*, 89: 143–186. (cited from Shimazu, '59) 1948.
10. Barron, D. H.: A note on the course of the proprioceptor fibers from the tongue. *Anat. Rec.*, 66: 11—15, 1936.
11. Batten, F. E.: The muscle spindle under pathological conditions. *Brain*, 20: 138–179, 1897.
12. Baum, J.: Beiträge zur Kenntnis der Muskelspindeln. *Anat. Hefte, I. Abt.*, 13: 249–305, 1900.
13. Bechterew, W. v.: Leitungsbahnen im Gehirn und Rückenmark. *Leipzig*. 1899.
14. Beck, W.: Ueber den Austritt des N. hypoglossus und N. cervicalis primus aus dem Centralorgan beim Menschen und in der Reihe der Säugetiere. *Anat. Hefte*, 6: 249–345, 1895.
15. Boecke, J.: Die morphologische Grundlage der sympathischen Innervation der quergestreiften Muskelfasern. *Z. f. mikro. anat. Forsch.*, 8: 561–639, 1927.
16. Boyd, I. A.: The innervation of mammalian neuromuscular spindles. *J. Physiol.*, 140: p. 14–15 (abstract) 1958.
17. Boyd, J. D.: Proprioceptice innervation of mammalian tongue. *J. Anat.*, 72: p. 147 (abstract) 1937.
18. Boyd, J. D.: The sensory component of the hypoglossal nerve in the rabbit. *J. Anat.*, 75: 330–345, 1941.
19. Bregmann, E.: Ueber experimentelle aufsteigende Degeneration motorischer und sensibler Hirnnerven. Arb. aus d. Inst. f. Anat. u. Physiol. d. Centralnervensystems a. d. Wiener Univ., 1: 73–97, 1892.
20. Bremer, L.: Uber die Endigungen der markhaltigen und marklosen Nerven im quergestreiften Muskel. *Arch. f. mikr. Anat.*, 21: 165–201, 1882.
21. Bremer, L.: Ueber die Muskelspindeln nebst Bemerkungen über Structur, Neubildung und Innervation der quergestreiften Muskelfaser. *Arch. f. mikr. Anat.*, 22: 318–356, 1883.
22. Brocklehurst, R. J., and F. H. Edgeworth: The fiber components of the laryngeal nerves of Macaca Mulatta. *J. Anat.*, 74: 386–389, 1940.
23. Brodal, A.: Central course of afferent fibers for pain in facial, glossopharyngeal and vagus nerves. Clinical observations. *Arch. Neur. & Psychiat.*, 57: 292–306, 1947.
24. Brodal, A.: Neurological anatomy in relation to clinical medicine. Oxford. 1948.
25. Bruesch, S. R.: The distribution of myelinated afferent fibers in the branches of the cat's facial nerve. *J. Comp. Neur.*, 81: 169–191, 1944.
26. Cajal, S. Ramón y: Histologie du système nerveux de l'homme & des vertébrés. Tome 1 (1952), Tome 2 (1955), Madrid. 1952, 55.

27. Carleton, A.: Observations on the problem of the proprioceptive innervation of the tongue. *J. Anat.*, 72: 502–507, 1938.
28. Carmichael, E. A., and H. H. Woollard: Some observations on the fifth and seventh cranial nerves. *Brain* 56: 109–125, 1933.
29. Cattaneo, A.: *Arch. Ital. de Biol.*, 10: 337–357, 1888. (cited from Hinsey, 1927) 1888.
30. Ciaccio, G. V.: *Arch. Ital. de Biol.*, 14: 31–57, 1891. (cited from Hinsey, 1927) 1891.
31. Cilimbaris, P. A.: Histologische Untersuchungen über die Muskelspindeln der Augenmuskeln. *Arch. f. mikr. Anat.*, 75: 692–747, 1910.
32. Cipollone, L. T.: *Suppl. Annuali di Midicina Navale*, 3: 282, 1897. (cited from Hines, 1927)
33. Clark, S. L.: Nissl granules of primary afferent neurons. *J. Comp. Neur.*, 41: 423–451, 1926.
34. Cogan, D. G.: Neurology of the ocular muscles. Oxford. 1948.
35. Cooper, S.: Muscle spindles in the intrinsic muscles of the human tongue. *J. Physiol.*, 122: 193–202, 1953.
36. Cooper, S., and P. M. Daniel: Muscle spindles in human extrinsic eye muscles. *Brain*, 72: 1–24, 1949.
37. Cooper, S., P. M. Daniel, and D. Whitteridge: The proprioceptive system of extraocular muscles. 4e Congrés Neurologique International Paris. Vol. 2, *Communications*, p. 70, 1949.
38. Cooper, S., P. M. Daniel, and D. Whitteridge: Cells of origin of proprioceptive fibres from eye-muscles. *J. Physiol.*, 111: p. 25 (abstract) 1950.
39. Cooper, S., P. M. Daniel, and D. Whitteridge: Afferent impulses in the oculomotor nerve, from the extrinsic eye muscles. *J. Physiol.*, 113: 463–474, 1951.
40. Cooper, S., P. M. Daniel, and D. Whitteridge: Nerve impulses in the brainstem of the goat. Short latency responses obtained by stretching the extrinsic eye muscles and the jaw muscles. *J. Physiol.*, 120: 471–490, 1953-a.
41. Cooper, S., P. M. Daniel, and D. Whitteridge: Nerve impulses in the brainstem of the goat. Responses with long latencies obtained by stretching the extrinsic eye muscles. *J. Physiol.*, 120: 491–513, 1953-b.
42. Cooper, S., P. M. Daniel, and D. Whitteridge: Nerve impulses in the brainstem and cortex of the goat. Spontaneous discharges and responses to visual and other afferent stimuli. *J. Physiol.*, 120: 514–527, 1953-c.
43. Cooper, S., P. M. Daniel, and D. Whitteridge: Muscle spindles and other sensory endings in the extrinsic eye muscles; The physiology and anatomy of these receptors and of their connections with the brainstem. *Brain*, 78: 564–683, 1955
44. Cooper, S., and M. Fillenz: Afferent discharges from the extrinsic eye muscles of the cat. *J. Physiol.*, 118: p. 49 (abstract) 1952.
45. Cooper, S., and M. Fillenz: Afferent discharges in response to stretch from the extraocular muscles of the cat and monkey and the innervation of these muscles. *J. Physiol.*, 127: 400–413, 1955.
46. Corbin, K. B.: Observations on the peripheral distribution of fibers arising in the mesencephalic nucleus of the fifth cranial nerve. *J. Comp. Neur.*, 73: 153–177, 1940.
47. Corbin, K. B., and F. Harrison: Further studies on tongue innervation. *Proc. Soc. for Exp. Biol. & Med.*, 38: 308–310, 1938-a.
48. Corbin, K. B., and F. Harrison: Proprioceptive components of cranial nerves. The spinal accessory nerve. *J. Comp. Neur.*, 69: 315–328, 1938-b.
49. Corbin, K. B., and F. Harrison: Function of the mesencephalic root of the fifth cranial nerve. *J. Neurophysiol.*, 3: 423–435, 1940.
50. Corbin, K. B., and F. Harrison: Further attempts to trace the origin of afferent nerves to the extrinsic eye muscles. *J. Comp. Neur.*, 77: 187–190, 1942.
51. Corbin, K. B., and J. C. Hinsey: Intramedullary course of the dorsal root fibers of each of the first four cervical nerves. *J. Comp. Neur.*, 63: 119—126, 1935.
52. Corbin, K. B., W. T. Lhamon, and D. W. Petit: Peripheral and central connections of the upper cervical dorsal root ganglia in the Rhesus monkey. *J. Comp. Neur.*, 66: 405–414, 1937.
53. Corbin, K. B., and R. K. Oliver: The origin of fibers to the grape-like endings in the insertion third of the extra-ocular muscles. *J. Comp. Neur.*, 77: 171–186, 1942.
54. Crevatin, F.: Ueber Muskelspindeln der Säugetieren. *Anat. Anz.*, 19: 173–176, 1901.
55. Daniel, P.: Spiral nerve endings in the extrinsic eye muscles of man. *J. Anat.*, 80: 189–193, 1946.
56. Darkschewitsch, L.: Ueber den Ursprung und den centralen Verlauf des Nervus accessorius Willisii. *Arch. Anat. Physiol., Anat. Abt. Jahrg.* 1885: 361–378, 1885.
57. Davis, L. E.: The deep sensibility of the face. *Arch. Neur. & Psychiat.*, 9: 283–305, 1923.
58. Deiters, O.: Unterschungen über Gehirn und Rückenmark des Menschen und der Säugethiere. *Braunschweig.* 1865.
59. Dogiel, A. S.: Die Nervenendigungen im Bauchfell, in den Schnen, den Muskelspindeln und den Centrum tendineum des Diaphragmas beim Menschen und bei Säugethieren. *Arch. f. mikr. Anat.*, 59: 1–31, 1902.
60. Dogiel, A. S.: Zur Frage über den fibrillären Bau der Sehnenspindeln oder der Golgi'schen Körperchen (organo nervoso terminale musculo-tendineo). *Arch. f. mikr. Anat.*, 67: 638–646, 1906-a.
61. Dogiel, A. S.: Die Endigungen der sensiblen Nerven in den Augenmuskeln und deren Sehnen beim Menschen und der Säugetieren. *Arch. f. mikr. Anat.*, 68: 501–526, 1906-b.
62. Downman, C. B. B.: Afferent fibres of the hypoglossal nerve. *J. Anat.*, 73: 387–395, 1939.
63. DuBois, F. S., and J. O. Foley: Experimental studies on the vagus and spinal accessory nerves in the cat. *Anat. Rec.*, 64: 285–307, 1936.
64. DuBois, F. S., and J. O. Foley: Quantitative studies of the vague nerve in the cat. II. The ratio of jugular to nodosal fibers. *J. Comp. Neur.*, 67: 69–87, 1937.

65. Duncan, D., and E. S. Crocker: Aberrant ganglion cells as a source of intact fibers in severed dorsal roots. *J. Neurophysiol.*, 2: 1–8, 1939.
66. Edinger, L.: Vorlesung über den Bau der nervösen Zentralorgane. Bd. 1, Leipzig. 1911.
67. Elliott, H. C.: *Textbook of the nervous system.* Philadelphia, London & Montreal. 1947.
68. Faaborg-Andersen, K.: Electromyographic investigation of intrinsic laryngeal muscles in humans. *Acta Physiol. Scand.*, 41: Suppl. 140, 1957.
69. Fahmy, N.: A note on the intracranial and extracranial parts of the ninth, tenth and eleventh nerves. *J. Anat.*, 61: 298–301, 1927.
70. Fillenz, M.: Responses in the brainstem of the cat to stretch of extrinsic ocular muscles. *J. Physiol.*, 128: 182–199, 1955.
71. Foley, J. O., and F. S. DuBois: An experimental study of the vagus nerve in the cat. *J. Comp. Neur.*, 60: 137–159, 1934.
72. Foley, J. O., and F. S. DuBois: Quantitative studies of the vagus nerve in the cat. I. The ratio of sensory to motor fibers. *J. Comp. Neur.*, 67: 49–67, 1937.
73. Foley, J. O., and F. S. DuBois: An experimental study of the facial nerve. *J. Comp. Neur.*, 79: 79–105, 1943.
74. Forster, L.: Zur Kenntniss der Muskelspindeln. Virchow's Arch., 137: 121–154, 1894.
75. Franqué, O. v.: Verh. phys. med. Gesell. Würzburg, N. F. 24: 1. (cited from Boyd, 1941) 1890.
76. Freeman, W.: The relationship of the radix mesencephalica trigemini to the extraocular muscles. *Arch. Neur. & Psychiat.*, 14: 111–113, 1925.
77. Freeman, W.: The columnar arrangement of the primary afferent centers in the brain-stem of man. *J. Nerv. & Ment. Dis.*, 65: 1–20, 149–170, 282–306, 378–397, 1927.
78. Friemann, R.: Untersuchungen über Zahl und Anordnung der Muskelspindeln in den Kaumuskeln des Menschen. *Anat. Anz.*, 100 (18/20). (cited from Winckler, 1956) 1954.
79. Froriep, A., and W. Beck: Ueber das Vorkommen dorsaler Hypoglossuswurzeln mit Ganglion, in der Reihe der Säugetiere. *Anat. Anz.*, 10: 688–696, 1895.
80. Fukuda, M.: Ganglion cells in the roots of III, IV and VI cranial nerves. (in Japanese) *Nippon Gakkai Zasshi*, 59: 958–970, 61: 283–291, 775–781, 1955, 57.
81. Fukuda, M.: Nerve endings in the extraocular muscles of the rabbit. (in Japanese) *Ibid.* 60: 318–334, 61: 51–61, 1955, 57.
82. Fukuda, M.: Studies on the ganglion cells in the roots of the 3rd, 4th and the 6th cranial nerves. *Jap. J. Ophth.*, 1: 60–65, 2: 42–47, 1957, 58.
83. Fukuda, M.: Studies on the nerve endings in the extrinsic eye muscles of the rabbit. *Jap. J. Ophth.*, 2: 93–102, 1958.
84. Garven, H. S. D.: The nerve-endings in the panniculus carnosus of the hedgehog, with special reference to the sympathetic innervation of striated muscle. *Brain*, 48: 380–441, 1925.
85. Gaskel, W. H.: On the relation between the structure, function, distribution and origin of the cranial nerves; together with a theory of the origin of the nervous system of vertebrata. *J. Physiol.*, 10: 153–211, 1889.
86. Gerard, M. W.: Afferent impulses of the trigeminal nerve. The intramedullary course of the painful, thermal and tactile impulses. *Arch. Neur. & Psychiat.*, 9: 306–338, 1923.
87. Giacomini, E.: *Accad. Fisiocrit., Siena*, Ser. 4, Vol. 10. (cited from Hinsey, 1827) 1898.
88. Goerttler, K.: Die Anordnung, Histologie und Histogenese der quergestreiften Muskulatur im menschlichen Stimmband. *Z. f. Anat. u. Entw.-gesch.*, 115: 352–401, 1950.
89. Golgi, C.: *Opera Omnia*, 1903, vol. 1. (cited from Hinsey, 1927) 1880-a.
90. Golgi, C.: Memor. della R. Accad. dell Sci. di Torino., Ser. II, Tomo 32. (cited from Huber & DeWitt, 1900) 1880-b.
91. Golgi, C.: *Arch. Ital. de Biol.*, 19. (cited from Pearson, 1943) 1893.
92. Golgi, C.: Ueber die Nerven der Sehnen des Menschen und anderer Wirbeltiere and über ein neues nervöses, musculotendinöses Endorgan. Untersuchungen über den feineren Bau des centralen und peripherischen Nervensystems. (übersetzt von Teuscher.) *Jena.* (p. 203) 1894-a.
93. Golgi, C.: Ueber den Ursprung des vierten Hirnnerven (patheticus oder trochlearis) und eine Frage der allgemeinen Histo-Physiologie, welche sich an diesen Gegenstand knüpft. *Ibidem.* (p. 261) 1894-b.
94. Grabower, B.: Ueber Nervenendigungen im menschlichen Muskel. *Arch. f. mikr. Anat.*, 60: 1–16, 1902.
95. Gregor, A.: Ueber die Vertheilung der Muskelspindeln in der Muskulatur des menschlichen Fötus. *Arch. f. Anat. u. Physiol., Anat. Abt. Jahrg.* 1904: 112–196, 1904.
96. Hagbarth, K. E., and G. Wohlfahrt: The number of muscle-spindles in certain muscles in cat in relation to the composition of the muscle nerves. *Acta Anat.*, 15: 85–104. (cited from Paulsen, 1958-b) 1952.
97. Hakamada, S.: Distribution of medullated nerve fibers in the monkey's leptomeninx and its blood vessels. (in Japanese) *Seishin-shinkeigaku Zasshi*, 47: 634–648, 1943.
98. Hamada, I.: Morphological study of nerve endings in the ocular muscles. (in Japanese) *J. Kyoto Prefect. med. Univ.*, 2: 1641–1678, 1928.
99. Hatano, S.: Studies on the muscle spindle. (in Japanese) *Tokyo J. med. Sci.*, 39: 433–521, 1925.
100. Head, H.: The afferent nervous system from a new aspect. *Brain*, 28: 99–115, 1905
101. Held, H.: Beiträge zur feineren Anatomie des Kleinhirns und Hirnstammes. *Arch. f. Anat. Physiol., Anat. Abt. Jahrg.* 1893: 435–446, 1893.
102. Henle, J.: Handbuch der systematischen Anatomie des Menschen. 2. Aufl., Bd. 3, Abt. 2, Braunschweig. 1879.
103. Hewer, E. E.: The development of nerve endings in the human foetus. *J. Anat.*, 69: 369–379, 1935.
104. Hines, M.: Nerve and muscle. *Quart. Rev. of Biol.*, 2: 149–180, 1927.
105. Hines, M.: Studies on the innervation of skeletal muscle. III. Innervation of the extrinsic eye muscles of the rabbit. *Am. J. Anat.*, 47: 1–53, 1931.

106. Hinsey, J. C.: Some observations on the innervation of skeletal muscles of the cat. *J. Comp. Neur.*, 44: 87–196, 1927.
107. Hinsey, J. C., and K. B. Corbin: Observations on the peripheral course of the secondary fibers in the first four cervical nerves of the cat. *J. Comp. Neur.*, 60: 37–44, 1934.
108. Holl, M.: Ueber den Nervus accessorius Willisii. *Arch. f. Anat. u. Physiol., Anat. Abt. Jahrg.* 1878: 491–517, 1878.
109. Hongo, T., K. Kubota, and H. Shimazu: Central inhibitory mechanism of spindle afferent discharge and brain activity. (in Japanese) *No to Shinkei* (Brain and Nerve), 11: 913–924, 1959.
110. Horsley, V.: Short note on sense organs in muscle and on the preservation of muscle spindles in conditions of extreme muscular atrophy, following section of the motor nerve. *Brain*, 20: 375–376, 1897.
111. Hosokawa, H.: On the extrinsic eye muscles of the whale, with special remarks upon the innervation and function of the musculus retractor bulbi. *Scient. Repts. Whales Res. Inst.*, No. 6: 1–33, 1951.
112. Huber, E.: Evolution of facial musculature and cutaneous field of trigeminus. Part III. *Quart. Rev. of Biol.*, 5: 389–437, 1930.
113. Huber, G. C.: A note on sensory nerve-endings in the extrinsic eye-muscles of the rabbit. "Atypical motor-endings" of Retzius. *Anat. Anz.*, 15: 335–342, 1899.
114. Huber, G. C.: Sensory nerve terminations in the tendons of the extrinsic eye-muscles of the cat. *J. Comp. Neur.*, 10: 152–158, 1900.
115. Huber, G. C., and L. M. DeWitt: A contribution on the motor nerve-endings in the muscle-spindles. *J. Comp. Neur.*, 7: 169–230, 1898.
116. Huber, G. C., and L. M. DeWitt: A contribution on the nerve terminations in neuro-tendinous end-organs. *J. Comp. Neur.*, 10: 159–208, 1900.
117. Huguenin, G.: Allgemeine Pathologie der Krankheiten des Nervensystems. I. Teil, Anatomische Einleitung. *Zürich.* (p. 263), 1873.
118. Hunt, J. R.: The sensory system of facial nerve and its symptomatology. *J. Nerv. & Ment. Dis.*, 36: 321–350, 1909.
119. Hunt, J. R.: Geniculate neuralgia (Neuralgia of the nervus facialis). *Arch. Neur. & Psychiat.*, 37: 253–285, 1937.
120. Inoue, T.: Studies of the muscle spindle in ocular muscles. (in Japanese) *Igaku Kenkyu* (Medical researches), 28: 4759–4773, 1958.
121. Ivy, R. H., and L. W. Johnson: Preservation of deep sensibility of the face after destruction of the fifth nerve. *Univ. of Penn. Med. Bull.*, 20: 35–38, 1907.
122. Johnston, J. B.: The radix mesencephalica trigemini. *J. Comp. Neur.*, 19: 593–664, 1909.
123. Kadanoff, D.: Die sensiblen Nervenendigungen in der mimischen Muskulatur des Menschen. *Z. mikr.-anat. Forsch.*, 62: 1–15, 1956.
124. Kazzander, J.: Ueber den Nervus accessorius Willisii und seine Beziehungen zu den oberen Cranialnerven beim Menschen und einiger Haussäugethieren. *Arch. f. Anat. Physiol., Anat. Abt. Jahrg.* 1891: 212–243, 1891.
125. Kerschner, L.: Bemerkungen über ein besonderes Nervensystem im willkürlichen Muskel. *Anat. Anz.*, 3: 126–132, 1888-a.
126. Kerschner, L.: Beitrag zur Kenntnis der sensiblen Endorgane. *Anat. Anz.*, 3: 288–296, 1888-b.
127. Kerschner, L.: Ueber die Fortschritte in der Erkenntnis der Muskelspindeln. *Anat. Anz.*, 8: 449–458, 1893.
128. Kiesewetter, G.: Untersuchungen über Zahl, Anordnung und Länge der Muskelspindeln in den Schultermuskeln des Menschen. *Anat. Anz.*, 106: 285–288, 1959.
129. Kimmel, D. L.: Development of the afferent components of the facial, glossopharyngeal and vagus nerves in the rabbit embryo. *J. Comp. Neur.*, 74: 447–471, 1941.
130. Kiss, F.: Anastomoses des nerfs de l'orbite. *Arch. Mus. Hist. nat. Paris*, 12: 239–242. (cited from Fillenz, 1955) 1935.
131. Kiyono, K.: Pathological changes of muscle spindle in the beriberi. (in Japanese) *J. Kyoto med. Assn.*, 8: 138–166, 193–222, 1911.
132. Kölliker, A.: Handbuch der Gewebelehre des Menschen. 6. Aufl. Bd. 2, Leipzig, 1896.
133. Körner, G.: Unterschungen über Zahl, Anordnung und Länge der Muskelspindeln in einigen Schulter-, den Oberarmmuskeln und im Muskulus sternalis des Menschen. *Anat. Anz.*, 108: 99–103, 1960.
134. Kosaka, K.: Zur Frage der physiologischen Natur der zerebralen Trigeminuswurzel. *Folia Neurobiol.*, 6: 1–16, 1912.
135. Krause, C. F. T.: Handbuch der menschlichen Anatomie. Bd. 1, (3. Aufl.) Hannover, 1876.
136. Krebs, P.: Die Nervenendigungen im Musculus stapedius mit besonderer Berücksichtigung der bei der Färbung angewandten Technik. *Arch. f. mikr. Anat.*, 65: 704—727, 1905.
137. Kühne, W.: Ueber die Endigung der Nerven in den Muskeln. *Virchow's Arch.*, 27: 508—533, 1863-a.
138. Kühne, W.: Die Muskelspindeln. *Virchow's Arch.*, 28: 528–538, 1863-b.
139. Kulchitsky, N.: Nerve endings in muscles *J. Anat.*, 58: 152–169, 1924.
140. Kulchitsky, N.: Nerve endings in the muscles of the frog. *J. Anat.*, 59: 1–17, 1925.
141. Kure, S.: Normale und pathologische Struktur der Zellen an der cerebralen Wurzel des N. trigemini. *Arb. aus. d. Inst. f. Anat. u. Physiol. d. Centralnervensystems a. d. Wierner Univ.*, 6: 158–181, 1899.
142. Langworthy, O. R.: Problems of tongue innervation; course of proprioceptive nerve fibers, autonomic innervation of skeletal masculature. *Johns Hopkins Hosp. Bull.*, 35: 239–246, 1924-a.
143. Langworthy, O. R.: A study of the innervation of the tongue musculature with particular reference to the proprioceptive mechanism. *J. Comp. Neur.*, 36: 273–298, 1924-b.
144. Larsell, O., and R. A. Fenton: The embryology and neurohistology of the sphenopalatine ganglion connections; a contribution to the study of otalgia. *Larnygoscope*, 38: 371—389, 1928.
145. Lewy, F. H., R. A. Groff, and F. C.

Grant: Autonomic innervation of the face. II. An experimental study. *Arch. Neur. & Psychiat.*, 39: 1238–1248, 1938.
146. Maloney, W. J., and R. F. Kennedy: The sense of pressure in the face, eye and tongue. *Brain*, 34: 1–28, 1911.
147. Marchi, V.: Ueber die Terminalorgane der Nerven (Golgi's Nervenkörperchen) in den Sehnen der Augenmuskeln. *Arch. f. Ophthalm.*, 28: I. Abt.: 203–213. 1882.
148. May, O., and V. Horsley: The mesencephalic root of the fifth nerve. *Brain*, 33: 175–203, 1910.
149. Mazzoni: Memor. della R. Acad. delle Sc. dell' instituto de Bologna. Serie quinta, T. 1. (cited from Hinsey, 1927), 1890.
150. McLean, A. J.: An attempt to locate cells of kinaesthetic sensibility in extra-ocular eye muscles. *Proc. Soc. Exp. Biol. Biol. & Med.*, 23: p. 658, 1926.
151. McLean, A. J.: An attempt to identify the central cells mediating kinesthetic sense in the extrinsic eye muscles. *Arch. Neur. & Psychiat.*, 17: 285–302, 1927.
152. Merkel, F.: Die trophische Wurzel des Trigeminus. *Unters. d. Anat. Inst. zu Rostock*, (cited from Golgi, 1894), 1874.
153. Merrillees, N. C. R.: The fine structure of muscle spindles in the lumbrical muscles of the rat. *J. Biophys. Biochem. Cytol.*, 7: 725–742, 1960.
154. Merrillees, N. C. R., S. Sunderland, and W. Hayhow: Neuromuscular spindles in the extraocular muscles in man. *Anat. Rec.*, 108: 23–30, 1950.
155. Meynert, Th.: Vom Gehirn der Säugethiere. Stricker's Handbuch der Lehre von den Geweben des Menschen und der Thiere. Bd. 2 (p. 747–748), Leipzig, 1871.
156. Mills, C. K.: The sensory function attempted to the seventh nerve. *J. Nerv. & Ment. Dis.*, 37: 273–284, 355–379, 1910.
157. Mingazzini, G.: Medulla oblongata und Brücke. Möllendorff's Handbuch d. mikr. Anat. d. Menschen. Bd. 4, 1. Teil, Berlin. (p. 579–643, 1928.
158. Mündnich, K.: Untersuchungen über den Bau der Nervenendigungen im Musculus vocalis und Musculus posticus des Kaninchenlarynx. *Z. f. Hals-Nasen u. Ohrenheilk.*, 41: 235–243, 1937.
159. Murray, J. G.: Innervation of the intrinsic muscles of the cat's larynx by the recurrent laryngeal nerve: a unimodal nerve. *J. Physiol.*, 135: 206—212, 1957.
160. Nakamura, N.: Histological observation of laryngeal muscles in the so-called sucker's beriberi. (in Japanese) *J. oto-rhino-laryng. Soc. Japan*, 21: 1156–1160, 1915.
161. Nakayama, H.: (cited from Kiyono, 1911), 1911.
162. Nakayama, M.: Nerve terminations in the muscle spindle of the human lingual muscles. (in Japanese) *Tohoku med. J.*, 34: 367–377, 1944.
163. Nicholls, G. E.: On the occurrence of an intracranial ganglion upon the oculomotor nerve in Scyllium canicula, with a suggestion as to its bearing upon the question of the segmental value of certain of the cranial nerves. *Proc. Roy. Soc. London*, Ser. B. 88: 553–568, 1915.
164. Nicholson, H.: On the presence of ganglion cells in the third and sixth nerves of man. *J. Comp. Neur.*, 37: 31–36, 1924.
165. Nozaki, K.: On the innervation, especially the sensory innervation of the periosteum and the area surrounding it in the earlier stage of human embryo. *Arch. Hist. Jap.*, 9: 269–282, 1956.
166. Obersteiner, H.: Anleitung bei Studium des Baues der nervösen. Centralorgane. 4. Aufl., Leipzig & Wien, 1901.
167. Okamoto, T.: Studies on the spiral endings found in nerves supplying human extrinsic ocular muscles. (in Japanese) *Acta Soc. ophthalmol. jap.*, 61: 2447–2466, 1957.
168. Omoto, S.: On the afferent neurons from the extra-ocular muscle receptors of rabbits. (in Japanese) *Ibid.*, 63: 1–8, 1959.
169. Oshima, T.: Anatomical studies of the muscle spindle. (in Japanese) *J. Nagasaki med. Assn.*, 16: 525–548, 1938.
170. Ota, N.: Studies on the nerve-endings of the middle ear muscles of the cat. (in Japanese) *J. oto-rhino-laryng. Soc. Japan*, 61: 1614–1630, 1958.
171. Pankratz, D. S.: The cranial-nerve components in the toad-fish (Opsanus tau.) *J. Comp. Neur.*, 50: 247–280, 1930.
172. Paulsen, K.: Untersuchungen über das Vorkommen und Zahl von Muskelspindeln im M. vocalis des Menschen. *Z. f. Zellforsch.*, 47: 363–366, 1958-a.
173. Paulsen, K.: Ueber Vorkommen und Zahl von Muskelspindeln in inneren Kehlkopfmuskeln des Menschen (M. cricoarytaenoideus dorsalis, M. cricothyreoideus). *Z. f. Zellforsch.*, 48: 349–355, 1958-b.
174. Pearson, A. A.: Observations on the spinal accessory nerve in human embryos. *Anat. Rec.*, 67: (suppl.) p. 39, 1937.
175. Pearson, A. A.: The spinal accessory nerve in human embryos. *J. Comp. Neur.*, 68: 243–266, 1938.
176. Pearson, A. A.: The trochlear nerve in human fetuses. *J. Comp. Neur.*, 78: 29–43, 1943-a.
177. Pearson, A. A.: Sensory type neurons in the hypoglossal nerve. *Anat. Rec.*, 85: 365–371, 1943-b.
178. Pearson, A. A.: The oculomotor nucleus in the human fetus. *J. Comp. Neur.*, 80: 47–63, 1944.
179. Pearson, A. A.: Further observations on the intramedullary sensory type neurons along the hypoglossal nerve. *J. Comp. Neur.*, 82: 93–100, 1945-a.
180. Pearson, A. A.: Observations on the roots of the facial nerve in human fetuses. *Anat. Rec.*, 91: 294–295. (abstract), 1945-b.
181. Pearson, A. A.: The development and connections of the mesencephalic root of the trigeminal nerve in man. *J. Comp. Neur.*, 90: 1–46, 1949.
182. Pearson, A. A., and S. A. O'Neill: A sliver-gelatin method for staining nerve fibers. *Anat. Rec.*, 95: 297–301, 1946.
183. Perrontito, A.: *Arch. Ital. de Biol.*, 38: 393–412. (cited from Hines, 1927), 1902.
184. Peters, G. A.: The presence of sensory nerve cells in the central root of the trigeminal nerve. *J. Comp. Neur.*, 62: 349–360, 1935.
185. Pilliet, A. H.: Sur les corpuscules neuro-musculaires a gaines pacinniennes. *J. de l' anat.*, 26: 602–616, 1890.
186. Ranson, S. W., and S. L. Clark: The anatomy of the nervous system. 8th ed., Philadelphia & London, 1951.

187. Ranson, S. W., H. K. Davenport, and E. A. Doles: Intramedullary course of the dorsal-root fibers of the first three cervical nerves. *J. Comp. Neur.*, 54: 1–12, 1932.
188. Retzius, G.: Zur Kenntniss der motorischen Nervenendigungen. Biol. Untersuchungen, Neue Folge, 3: 41–52, 1892.
189. Rhinehart, D. A.: The nervus facialis in the albino mouse. *J. Comp. Neur.*, 30: 81–125, 1918.
190. Rogers, K. T.: Ocular muscle proprioceptive neurons in the developing chick. *J. Comp. Neur.*, 107: 427–437, 1957.
191. Ruffini, A.: Considerazioni critiche sui recenti studi dell'apparato nervoso nei fusi muscolari. *Anat. Anz.*, 9: 80–88, 1894.
192. Ruffini, A.: Observations on sensory nerve-endings in voluntary muscles. *Brain*, 20: 368–374, 1897.
193. Ruffini, A.: On the minute anatomy of the neuromuscular spindles of the cat, and on their physiological significance. *J. Physiol.*, 23: 190—208, 1898.
194. Sano, F.: Ueber die zwei- oder mehrfache Teilung des Trochleariskerns beim Menschen und einigen Säugetieren. (in Japanese) *Tokyo J. med. Sci.*, 55: 490–497, 1941.
195. Sano, K.: Wartenberg's "The Examination of Reflexes." (translated into Japanese) Tokyo, 1953.
196. Sano, K.: Trigemino-oculomotor synkinesis. Neurologia medico-chirurgica, 1: 29–51, 1959.
197. Schaffer, J.: Lehrbuch der Histologie und Histogenese. 3. Aufl., Berlin & Wien, 1933.
198. Schneider, A. J.: The histology of the radix mesencephalica nervi trigemini in the dog. *Anat. Rec.*, 38: 321–339, 1928.
199. Schulze, M. L.: *Anat. Anz.*, 102. (cited from Kiesewetter, 1958), 1955.
200. Schwalbe, G.: Lehrbuch der Neurologie. C. E. E. Hoffmann's Lehrbuch der Anatomie des Menschen. 2. Bd. Erlangen, 1881.
201. Seto, H.: Sensory innervation of motor organs. (in Japanese) The sensory innervation of human body. (p. 52) Tokyo, 1957.
202. Sheinin, J. J.: Typing of the cells of the mesencephalic nucleus of the trigeminal nerve in the dog, based on nisslgranule arrangement. *J. Comp.Neur*, 50: 190–131, 1930.
203. Sheinin, J. J.: Studies of the mesencephalic nucleus in the normal and experimental cat. *Anat. Rec.*, 55: p. 36. (abstract), 1933.
204. Sherrington, C. S.: On the anatomical constitution of nerves of skeletal muscles; with remarks on recurrent fibers in the ventral spinal nerve-root. *J. Physiol.*, 17: 211–258, 1894.
205. Sherrington, C. S.: Further note on the sensory nerves of the muscles. *Proc. Roy. Soc. London*, Ser. B. 61: 247–249, 1897.
206. Sherrington, C. S.: Further note on the sensory nerves of the eye muscles. *Proc. Roy. Soc. London*, Ser. B. 64: 120–121, 1898.
207. Sherrington, C. S.: Observations on the sensual role of the proprioceptive nerve-supply of the extrinsic ocular muscles. *Brain*, 41: 332–343, 1918.
208. Shimazu, H.: Physiology of γ system. (in Japanese) *Shinkei Kenkyu No Shinpo* (Recent advances in the neurological research), 3: 781–846, 1959.
209. Shimoda, F.: Innervation, especially sensory innervation of the knee-joint and the motor organs around it in early stage of human embryo. *Arch. Hist. Jap.*, 9: 91–107, 1956.
210. Siemerling, E.: Ein Fall von gummöser Erkrankung der Hirnbasis mit Betheiligung des Chiasma nervorum opticum. *Arch. f. Psychiat.*, 19: 401–437, 1888.
211. Sinclair, J. G.: A developmental study of the fourth cranial nerve. *Texas Rep. Biol. Med.*, 16: 253–267, 1958.
212. Slawik, F. E.: Ueber das Vorkommen von Muskelspindeln in der Musculus propria des menschlichen Oesophagus. *Anat. Anz.*, 93: 133–137. (cited from Stöhr, 1957), 1942.
213. Smith, O. C.: Personal communication, (cited from Hines, 1927), 1926.
214. Smyth, G. E.: The systemization and central connections of the spinal tract and nucleus of the trigeminal nerve. A clinical and pathological study. *Brain*, 62: 41–87, 1939.
215. Spiller, W. G.: *J. Nerv. & Ment. Dis.*, 33: p. 736. (abstract), 1906.
216. Stibbe, E. P.: Sensory components of the motor nerves of the eye. *J. Anat.* 64: p. 112. (abstract), 1929.
217. Stilling, B.: Untersuchungen über den Bau des Hirnknotens. Jena, 1846.
218. Stöhr, P., Jr.: Das peripherische Nervensystem. Möllendorff's Handbuch d. mikr. Anat. d. Menschen. 4. Bd., 1. Teil. Berlin. (p. 228), 1928.
219. Stöhr, P., Jr.: Mikroskopiche Anatomie des vegetativen Nervensystems. Möllendorff's Handbuch. 4. Bd., 5. Teil. Berlin, Göttingen & Heidelberg, 1957.
220. Streeter, G. L.: The development of the cranial and spinal nerves in the occipital region of the human embryo. *Am. J. Anat.*, 4: 83–116, 1905.
221. Strong, H.: Neuromuscular-like-fibers of the vocal ligament. *Anat. Rec.*, 91: p. 302. (abstract), 1945.
222. Sugano, M.: On the laryngeal nerves. (in Japanese) *J. oto-rhino-laryng. Soc. Japan*, 35: 1338–1361, 1362–1375, 1386–1400, 1929.
223. Sunderland, S.: A preliminary note on the presence of neuromuscular spindles in the extrinsic ocular muscles in man. *Anat. Rec.*, 103: 561–562, 1949.
224. Sunderland, S., and E. S. R. Hughes: The pupillo-constrictor pathway and the nerves to the ocular muscles in man. *Brain*, 69: 301–309, 1946.
225. Sunder-Plassmann, P.: Ueber den Nervenapparat des Musculus vocalis. Z. f. Hals-Nasen u. Ohrenheilk, 32: 493–499, 1933-a.
226. Sunder-Plassmann, P.: Ueber den Nervenapparat des menschlichen Glottisöffners, Musculus crico-arytaenoideus posticus. Z. f. Hals- Nasen u. Ohrenheilk., 32: 586–598, 1933-b.
227. Sutton, A. C.: On the development of the neuro-muscular spindle in extrinsic eye muscles of the pig. *Am. J. Anat.*, 18: 117–144, 1915.
228. Takasaki, T.: Motor nerve endings in the dog's vocal muscle. (in Japanese) *J. oto-rhino-laryng. Soc. Japan*, 34: 676–679, 1928.
229. Tarkhan, A. A.: The innervation of the extrinsic ocular muscles. *J. Anat.*, 68: 293–313, 1934.

230. Tarkhan, A. A.: Ein experimenteller Beitrag zur Kenntnis der proprioceptive Innervation der Zunge. *Z. Anat. u. Entw. gesch.*, 105: 349–358, 1936-a.
231. Tarkhan, A. A.: Ueber das Vorhandensein afferenter Fasern in Nervus hypoglossus. *Arch. Psychiat. u. Nervenkr.*, 105: 475–483, 1936-b.
232. Tarkhan, A. A., and S. Abd El-Malek: On the presence of sensory nerve cells on the hypoglossal nerve. *J. Comp. Neur.*, 93: 219–228, 1950.
233. Tarkhan, A. A., and Abou-El-Naga I: Sensory fibers in the hypoglossal nerve. *J. Anat.*, 81: 23–32, 1947.
234. Tarlov, I. M.: Sensory and motor roots of the glossopharyngeal nerve and the vagus-spinal accessory complex. *Arch. Neur. & Psychiat.*, 44: 1018–1021, 1940.
235. Tello, J. F.: Die Entstehung der motorischen und sensiblen Nervenendigungen. I. In dem lokomotorischen Systeme der höheren Wirbeltiere. Muskuläre Histogenese. *Z. f. Anat. u. Entwgesch.*, 64: 348–440, 1922.
236. Terterianz, M.: Die obere Trigeminuswurzel. *Arch. f. mikr. Anat.*, 53: 632–659, 1899.
237. Thanhoffer, L. v.: Ueber die Nervenendigung der quergestreiften Muskelfasern und über Re- und Degeneration derselben im lebenden Körper. *Anat. Anz.*, 7: 635–638, 1892.
238. Thelander, H. E.: The course and distribution of the radix mesencephalica trigemini in the cat. *J. Comp. Neur.*, 37: 207–220, 1924.
239. Thomsen, R.: Ueber eigenthümliche aus veränderten Ganglienzellen hervorgeganene Gebilde in den Stämmen der Hirnnerven des Menschen. *Virchow's Arch.*, 109: 459–465, 1887.
240. Tiegs, O. W.: Innervation of voluntary muscle. *Physiol. Rev.*, 33: 90–144, 1953.
241. Tomasch, J., and W. A. Britton: A fibre-analysis of the laryngeal nerve-supply in man. *Acta Anat.*, 23: 386–398, 1955.
242. Tozer, F. M.: On the presence of ganglion cells in the roots of III, IV and VI cranial nerves. *J. Physiol.*, 45: p. 15–16, 1912.
243. Tozer, F. M., and C. S. Sherrington: Receptors and afferents of the third, fourth, and sixth cranial nerves. *Proc. Roy. Soc. London*, Ser. B, 82: 450–457, 1910.
244. Tschiriew, A.: Sur les terminaisons nerveuses dans les muscles striées. *Arch. de Physiol., Sec.* Ser. 6: 91–116. (cited from Hinsey, 1927), 1879.
245. Van der Sprenkel, H. B.: The hypoglossal nerve in an embryo of Erinaceus europaeus. *J. Comp. Neur.*, 36: 219–270, 1924.
246. Van Gehuchten, A.: Anatomie du système nerveus de l'homme. Louvain, 1906.
247. Van Valkenburg, C. T.: Zur vergleichenden Anatomie des mesencephalen Trigeminusanteils. *Folia Neurobiol.*, 5: 360–418, 1911.
248. Vogt, H.: Feinere Anatomie der Medulla oblongata, des Kleinhirns, der Brücke und des Mittelhirns. *Lewandowsky's Handbuch d. Neur.*, 1. Bd., Berlin, 1910.
249. Voss, H.: Untersuchungen über Zahl, Anordnung und Länge der Muskelspindeln in den Lumbricalmuskeln des Menschen und einiger Tiere. *Z. f. mikr.-anat. Forsch.*, 42: 509–524, 1937.
250. Voss, H.: Beiträge zur mikroskopischen Anatomie der Augenmuskeln des Menschen. (Faserdicke, Muskelspindeln, Ringbinden.) *Anat. Anz.*, 104: 345–355, 1957.
251. Voss, H.: Zahl und Anordnung der Muskelspindeln in den unteren Zungenbeinmuskeln, dem M. sternocleidomastoideus und den Bauch-und tiefen Nackenmuskeln. *Anat. Anz.*, 105: 265–275, 1958.
252. Wakabayashi, I.: Functional combination between the muscle and nerve (in Japanese). *J. Hiroshima Med. Assn.*, 10: 590–595, 1958.
253. Wakeley, C. P. G., and F. H. Edgeworth: A note on the afferent nerve-supply of the facial muscles. *J. Anat.*, 67: 420–421, 1933.
254. Walker, A. E.: The origin, course and terminations of the secondary pathways of the trigeminal nerve in primates. *J. Comp. Neur.*, 71: 59–89, 1939.
255. Wallenberg, A.: Sekundäre Bahnen aus dem frontalen sensibeln Trigeminuskerne des Kaninchens. *Anat. Anz.*, 26: 145–155, 1905.
256. Wartenberg, R.: The examination of reflexes (translated by K. Sano into Japanese), 1945.
257. Weddell, G., J. A. Harpman, D. G. Lambley, and L. Young: The innervation of the musculature of the tongue. *J. Anat.*, 74: 255–267, 1940.
258. Weigner, K.: Beziehungen des Nervus accessorius zu den proximalen Spinalnerven. *Anat. Hefte*, (Abt. I) 17: 551–587, 1901.
259. Weigner, K.: Ueber den Verlauf des Nervus intermedius. *Anat. Hefte*, 29: 97–162, 1905.
260. Weinberg, E.: The mesencephalic root of the fifth nerve. A comparative anatomical study. *J. Comp. Neur.*, 46: 249–405, 1928.
261. Whitteridge, D.: *Quart. J. Exp. Physiol.*, 40: 331 (cited from Cooper), Daniel, and Whitteridge, 1955), 1955.
262. Willard, W. A., and J. C. Lawrence: Some observations on the spinal accessory and cervical nerves in the innervation of the sternocleidomastoid muscle in the mouse. *Anat. Rec.*, 55:p. 82. (abstract), 1933.
263. Willems, E.: Les noyaux masticateur et mésencéphalique du trijumeau chez la lapin. *Nevraze*, 12: 1–220. (cited from Corbin, 1940), 1911.
264. Winckler, G.: L'innervation sensitive et motrice des muscles extrinsiques de l'oeil chez quelques ongules. *Arch. Anat.*, Strasbourg, 23: 219–234. (cited from Fillenz, 1955), 1937.
265. Winckler, G.: Les différents critères d'innervation proprioceptive des muscles du larynx chez l'homme. *Arch. Anat.*, 33: 1, 3–5. (cited from Paulsen, 1958–b), 1956.
266. Winckler, G.: L'innervation proprioceptive des muscles extrinsèques du globe oculaire chez le bouquetin et le chevreuil. *Okajima's Folio anat. jap.*, 28: 341–351, 1956.
267. Windle, W. F.: Non-bifurcating nerve fibers of the trigeminal nerve. *J. Comp. Neur.*, 40: 229–240, 1926.
268. Windle, W. F.: The neurofibrillar structure of the spinal cord of cat embryos correlated with the appearance of early

somatic movements. *J. Comp. Neur.*, 53: 71–113, 1931-a.

269. Windle, W. F.: The sensory components of the spinal accessory nerve. *J. Comp. Neur.*, 53: 115–127, 1931-b.
270. Windle, W. F.: Neurons of the sensory type in the ventral roots of man and other mammals. *Arch. Neur. & Psychiat.*, 26: 791–800, 1931-c.
271. Windle, W. F.: The neurofibrillar structure of the 7-mm cat embryo. *J. Comp. Neur.*, 55: 99–138, 1932.
272. Windle, W. F., and L. C. De Lozier: The absence of painful sensation in the cat during stimulation of the spinal accessory nerve. *J. Comp. Neur.*, 54: 97–101, 1932.
273. Wolff, E.: *The anatomy of the eye and orbit*. 4. ed., London, 1958.
274. Wolter, J. R.: Ueber Nervenendigungen in der äusseren Augenmuskulatur. *Acta Neurovegetativa*, 4: 343–353, 1952.
275. Wolter, J. R.: Morphology of the sensory nerve apparatus in striated muscle of the human eye. *Arch. Ophthal.*, 53: 201–207, 1955.
276. Woollard, H. H.: The innervation of the ocular muscles. *J. Anat.*, 65: 215–223, 1931.
277. Yee, J., and K. B. Corbin: The intramedullary course of the upper five, cervical, dorsal root fibers in the rabbit. *J. Comp. Neur.*, 70: 297–304, 1939.
278. Yee, J., F. Harrison, and K. B. Corbin: The sensory innervation of the spinal accessory and tongue musculature in the rabbit. *J. Comp. Neur.*, 70: 305–314, 1939.
279. Yokomatsu, K.: Studies of nerves and nerve endings in the visual organs. VI. Nerve endings in the extrinsic eye muscles (in Japanese). *Acta anat. nippon.*, 4: 1331–1354, 1932.
280. Youngstrom, K. A.: Intramedullary sensory type ganglion cells in the spinal cord of human embryos. *J. Comp. Neur.*, 81: 47–53, 1944.

CANCER OF THE LUNG 1930 TO 1960: A REVIEW[a]

R. H. RIGDON AND HELEN KIRCHOFF[b]

One hundred years ago cancer of the lung was practically unknown. Fifty years ago it was a rare disease. Today this cancer has the highest neoplastic mortality rate in the male. As Oniugbo (304) so aptly wrote in his review of lung cancer in the 19th century, "Lung cancer is today a common disease of uncommon interest." More has been written about it in scientific literature and the lay press than any other neoplastic lesion.

Cancer of the lung is a worldwide disease as indicated by published articles from Austria (83, 217, 366); Australia (60, 445); Argentina (117, 244); Belgium (82); China (184); Canada (40, 209, 267); Czechoslovakia (195); Denmark (62, 63); England (31, 36, 199, 200, 201, 314, 408); Finland (216); France (84, 127, 224, 226, 361); Greece (325); Germany (123, 145, 238, 239, 281, 333, 370, 455); Iceland (108); India (131); Italy (81, 236, 237, 322, 428); Japan (285); Mexico (2); the Netherlands (211, 212, 291); Norway (218, 219); the Philippines (385); Portugal (421); Pakistan (190); Russia (380); Sweden (353); Switzerland (146, 147); South Africa (130); and, the United States (45, 54, 98, 99, 100, 124, 260, 297, 356, 402, 403, 438). However, the frequency of death from cancer of the lung differs in different countries as shown by data compiled in 1955 by the World Health Organization (313). The incidence rates differ as shown by the study of 10 cities in the United States for the years 1938 to 1948 (54). No pulmonary carcinomas were observed in autopsy studies in South Africa in 1934 (412), Ceylon in 1944 (69), or Trinidad in 1945 (376), but observations made in South Africa in 1949 (130) showed the presence of this cancer.

Bayle (22), a Frenchman, was the first to report a case of cancer of the lung in 1810. In 1851 Storer (411) published the first case in the United States. By 1893, 132 cases had been collected

[a] This study was aided by a grant from the Tobacco Industry Research Committee.

[b] From the Department of Pathology, The University of Texas Medical Branch, Galveston, Texas. Submitted for publication April 5, 1961.

from the literature (339), but of these Passler (319) accepted only 70 as authentic. From 1900 to 1930 many statistical studies based on autopsies were reported and all showed that cancer of the lung was progressively increasing (340). In 1927 Weller (441) referred to the increase as "epidemic." By 1930 no one could deny that cancer of the lung was on the increase—but was this a real or only apparent increase? Investigators had tried to establish this point before 1930 (339) and in 1930 Homann (180) reviewed the available literature and concluded that the increase was only apparent. In 1933 Hamman (159) said there were not sufficient data to settle the dispute and noted that two authors, Rosahn and Fried, using the same statistical material, arrived at opposite conclusions. Rosahn wrote that the postmortem incidence of primary carcinoma of the lung was steadily increasing and the increase was real and absolute, while Fried stated that the increase was very likely more apparent than real. Sweany (414) in 1934 thought that much of the tenfold increase in lung cancer during the past 50 years could be accounted for by the increased life expectancy, the increased knowledge of certain diseases, such as tuberculosis and bronchiectasis, the tremendous effect X-ray and the bronchoscope had on diagnosis, the increased zeal on the part of the medical profession and laity, and the pathologist's changing attitude towards metastases and sarcomas. Fried (125) also thought the increase might be due to progress in hygiene and preventive medicine resulting in increased longevity. In 1935 Neely (288) said that it was very likely that the increase was more apparent than real and probably due to a combination of factors.

In 1937 Frissell and Knox (126), based on data from autopsies, also concluded that the increase in lung cancer probably was apparent rather than real. Jaffe (193) concurred in this opinion. El-Gazayerli (111) in 1936 thought it premature to say whether the increase of lung cancer was real or only apparent. Klotz (209) in 1938 wrote that much of the increase was apparent. In 1943 Johnson and Reinhart (196) found 66 lung cancer cases in 8,333 autopsies performed during the years 1917 to 1940; they felt the increase was "more relative than absolute" even though their data showed only four lung cancer cases for the years 1917 to 1922 and 37 for the years 1937 to 1942.

Better diagnosis and the aging of the population were the cause of the increase in lung cancer according to Macklin (253) and Peery (321) in 1940. Dorn (98) in 1943 said, "The reported increase in the death rate from respiratory cancer is due primarily to an increase in late adult life." From a study of the incidence of lung cancer in Denmark made in 1948, Clemmesen and Busk (62) thought the more frequent detection of the disease was due to the improvement of diagnostic procedures and increased attention. In 1948 McEwen (264) considered the aging population the greatest factor in the increase of this neoplasm in Canada. In the same year Fried (124) restated his opinion that the increase was in all likelihood more apparent than real.

On the other side of the controversy, Craver (72) in 1935 felt that cancer of the lung was actually on the increase. In 1937 Simons (384) wrote, "The incidence of the disease has increased both absolutely and relatively." Menne and Anderson (271) in 1941 found an absolute increase in their study made in the Pacific Northwest. In the same year Ochsner and DeBakey (297) stated that the frequency of lung cancer was absolutely increasing. Some of the other writers who felt that the increase was real were Rosahn (357) in 1930, Wallace and Jackson (432) in 1943, Mandell (256) in 1947, McConnell *et al.* (261) in 1952, and Rakov (332) in 1957.

Rosenblatt and Lisa (359), writing in 1956, said there "has been a dramatic upswing since 1925 in the incidence of lung cancer but it is not realistic to compare incidence statistics of the present with those of three decades ago, when the means to diagnose the disease were not available. Although much of the increased incidence of lung cancer is relative, *i.e.*, due to improved diagnostic methods, a considerable part of the increase is real. Lung cancer is a disease of late life, and the number of potential candidates is steadily increasing. In 1900 there were only three million people aged 65 or over in the United States, while at present there are 13 million. It is estimated that the number will reach 20 million by 1975. . . . The incidence of lung cancer apparently increased to a greater extent than other cancers because of its more recent recognition. After a period of years its rate of increase will undoubtedly become smaller."

The question—Is cancer of the lung actually on the increase,

and if so to what extent?—remains unanswered. Dorn (101) in 1954 said there were no reliable data related to it and the answer must be largely a matter of opinion. McKenzie (265) stated in 1956 that it could not be answered in retrospect. Discussing this in 1959 Cornfield *et al.* (70) pointed out, "The vital statistics and the necropsy data that support the presumption of a real increase in lung-cancer risk certainly apply to the years after 1920. Because of the uncertainties associated with changes in diagnostic accuracy, no firm conclusions can be reached on whether the rate of increase in lung-cancer mortality has, in truth, accelerated since 1920."

"Nowhere," said Macklin (254) in 1948, "has there been more muddled thinking than on the question of whether lung cancer has increased or not. The question has been impossible to answer because we have no standard comparison." It is not difficult to understand this confusion when we remember that the basic data of mortality incidence come from death certificates (102). In 1936 Kennaway and Kennaway (202) warned against the use of death certificates for statistical purposes. Although Bonser and Thomas (37) found only a small error in the certification of lung cancers in a study made in Leeds in 1955, they concluded. "The reasons for the errors are partly due to ambiguity of wording of the certificates, partly to mis-diagnosis and partly to the intervention of other causes of death."

It was 1933 before we had nationwide death reporting in this country and 1939 before bronchogenic carcinoma was listed as such in our vital statistics. Gilliam (134), Chief of the Epidemiology Section of the National Cancer Institute, pointed out in 1955, "The International Lists of Causes of Death did not separately list cancer of the lung and pleura, or even cancer of the respiratory system as a whole, until the Fourth Revision—first used in 1930. . . . Prior to 1930 the term bronchogenic carcinoma was lumped with 90 other cancers of various sites. Between 1930 and 1938, when this diagnosis appeared on a death certificate, it was tabulated with cancer and other malignant tumors of other respiratory organs (47C) that is, with tumors other than those designated as larynx (47A) or as lungs and pleura (47B). It was not until the Fifth Revision came into use in 1939 that deaths which physicians charged to bronchogenic carcinoma were sep-

arately identified in official statistics." According to Milmore (276) of the United States Public Health Service, it is impossible to obtain the number of deaths attributed to bronchogenic carcinoma prior to 1939. Prior to 1930 it is not even possible to obtain the total deaths from bronchogenic carcinoma plus any combination of respiratory system cancers. This, of course, would magnify the apparent recorded increase in lung cancer mortality from 1939 to the present time when compared with the years before 1939.

Regardless of whether the increase is only apparent or real, in 1960 cancer of the lung ranked first as the cause of death from cancer among men. Clemmesen, according to Gsell (146), expressed the opinion that there will be a sixfold increase of bronchogenic carcinoma for the year 1990 based upon the data of 1950. However, Dorn's (99) statistical studies reported in 1953 indicated that the increase in mortality from lung cancer was slowing down. Between 1940 and 1950 the rate of increase in mortality declined and was only one-half that of the previous decade. Gilliam (134) reported in 1955, "I have previously stated that the rate of increase in recorded mortality was greatest in this country between 1914 and 1930 and that it has been declining since." Haenszel and Shimkin (155) concluded from a study made in 1956 that there would be a slowing down of the past lung cancer rate by 1965 and 1975.

Etiology

As cancer of the lung increased, the search for its etiology was intensified. Before 1900 lung cancer was associated with such factors as tuberculosis, syphilis, pulmonary scarring and heredity (339) and Bruen (49) in 1885 suggested irritation as a cause. In 1892 Osler (309) commented on the high incidence of this cancer among the cobalt miners. At the beginning of the 20th century exogenous agents were beginnig to be considered seriously and these were discussed in our review of lung cancer from 1900 to 1930 (340). Cancer of the lung was observed to be more frequent among certain industrial workers, but the total number of such cases accounted for only a small proportion of the increase in pulmonary neoplasms.

Lehmann (228) in 1932 apparently was the first to report the occurrence of lung cancer among *chromate workers*. Additional

observations support this association (18, 30, 46, 144, 185, 252, 324). The only form of lung cancer officially recognized in Great Britain as an industrial disease is that caused by *nickel* produced by the decomposition of a gaseous nickel compound (85). Other investigators have reported pulmonary cancer in nickel workers (58, 86, 138, 245). Uhlig (425) in 1921 apparently was the first to suggest that the higher incidence of lung cancer in the Schneeberger miners was due to the *radioactivity of the ore*. Others have concurred in the opinion of the role of radioactive ores in the production of pulmonary cancers (186, 187, 323, 383). In Japan, workers at *illuminating gas generators* have shown a high incidence of lung cancer (198, 222). Similar observations have been made in England (87, 201). The first case associated with asbestosis was reported in the United States in 1935 by Lynch and Smith (250). Similar cases have been observed in England (88, 272, 391) and in the United States (449). Wegelius (440), however, did not find any cases of lung cancer associated with asbestosis in Finland.

There is some suggestion that *iron workers* under certain conditions may have a high incidence of lung cancer (44, 279, 334, 423, 430, 450). However, Doll (89) failed to find any relation between "hot metal" workers and the frequency of lung cancer. There is considerable controversy as to whether cancer of the lung may be a manifestation of *arsenic poisoning* (175, 188, 392, 400). Vallee *et al.* (427) in 1960 stated, "The production of arsenical cancer in animals has never been satisfactorily demonstrated." Kennaway and Kennaway (201) in 1947 observed a high mortality from lung cancer in men exposed to dust from *tarred roads* and *exhaust from motor vehicles*. Such a relation, however, was not observed by Wynder and Graham (450), Doll (89), or Raffle (331). The first case of lung cancer associated with *berylliosis* was reported from Germany in 1954 (197). Less frequent observations on lung cancer have been made on metal grinders (202), or in association with pneumoconiosis (188) and printers' ink (13).

The role of smoking in lung cancer, although suggested before 1900, continued to be the burning question between 1930 and 1960. Lickint (240) in 1930 and Hoffman (177) in 1931 cited tobacco as the etiology of lung cancer. In 1932 Brockbank (48)

pointed out that ordinary home dust, road and trade dusts, tar petrol, radioactive gases, gaseous arsenical compounds, irritant vapors in chemical plants and laboratories, poison gases and tobacco smoke had been cited as etiological factors, but in his opinion only tuberculosis, influenza, chronic bronchitis and inhalation of irritant substances warranted serious consideration. Boycott (39) felt "environment as a whole had become less carcinogenic but that there were more tarred roads, more petrol engines being used, and more tobacco smoked, and whatever the agent, it produced lung cancer more frequently in men than women."

McNally (266) in 1932 called attention to the fact that the increase in lung cancer paralleled the increase in the consumption of cigarettes and said "because of this, one is certainly led to believe that cigarette smoking is an important factor in the increase in the frequency of the disease." However, in 1934, Sweany (414) said, "If there is any merit in this theory it should be manifest with the female sex, where the increase in the use of cigarettes has been greater than in men. If, for example, the ratio of men to women 20 years ago was 3 to 1 and now it is 1.5 to 1, the case is well nigh proved; so far no such increase in ratios has appeared. The sex ratio was, and still is, around 3 males to 1 female, similar to other malignant conditions other than those of sex difference. On the other hand, there are many patients with lung cancer who do not give a history of cigarette smoking at all." In 1957, 27 years later, Clough (65) said that a leveling off of the lung cancer rate in man and a rapid increase in females could be expected during the next two decades if the apparent carcinogenic effect of smoking is real. Rosenblatt and Lisa (359) in 1956 stated, "One of the arguments put forth in favor of the smoking theory is the prevalence of the disease in males, attributing the sex difference to their heavier smoking habits. Although the incidence of smoking among females has steadily increased since World War I, the ratio of lung cancer between sexes is still predominantly in favor of the males. This has occurred despite the increased incidence of the disease in women. If smoking were of etiological significance there should have resulted some decrease in the sex ratio over a period of years. However, recent statistical studies show the ratio to have actually increased."

Fried (124) commented, "The peculiar selectivity of bronchiogenic cancer for the male has not been adequately explained. Some authorities incriminate smoking of cigarettes. This explanation, though attractive for its simplicity, is hardly plausible."

In 1933 Ferrari (116) thought the carcinogenic properties of the tar in tobacco smoke might account for the higher incidence in men, but Hruby and Sweany (183) thought that while disease, smoke, dust and modes of living might have been the cause of some of the increase in lung cancer, better diagnosis and changed conditions contributed the most. In 1936 Arkin and Wagner (11) said inhalation of tobacco smoke was an important factor in producing chronic irritation and both Fleckseder (123) and Roffo (355) attributed the high percentage of lung cancer in men to smoking. However, Passey and Holmes (314) reported that their experimental work did not support the claim that tobacco smoke was an etiological factor and asked, "If in reality this was the main determining cause of the so-called increase in lung cancer, how can the supporters of this theory explain the fact that cancer of the tongue, larynx and pharynx have not increased in proportion to the increase which they claim to detect in the case of the lung, which after all, is the last part of the respiratory tract to be exposed to the influence?" Craver (72) noted that the apparent actual increase of cancer of the lung had been attributed to a change in the quality of atmospheric dusts filled with combustion products of gasoline and particles from tarred roads and stated that the effects of respiratory infections, chronic bronchitis and bronchiectasis and possibly influenza were also possible factors. Kennaway (202) made a study of the comparative incidence of lung cancer in various occupations and found that workers exposed to coal gas and tar and those engaged in the preparation and sale of tobacco tended to show an increased prevalence of cancer of the lung, but "no special occupations have been found to which the increase in the total cases of cancer of the lung can be attributed; rural workers show an increase which is not much less than that in the general population. . . . Coal tar in the atmosphere does not cause an exceptionally high incidence of cancer of the lung."

In 1937 Simons (384) wrote, "Tobacco smoke as a lung irri-

tant may be considered among possible causes of pulmonary carcinoma but cannot be counted an important one in the present state of information." Along the same vein, an editorial in a 1937 issue of the Journal of the American Medical Association (109) said that the injurious effects of tobacco smoking, exhaust gases from automobiles, tar on roads and the influenza epidemic of 1918 to 1919 have been accused of contributing to the increased incidence of lung cancer but "none of these can be accepted as definitely causative." In 1938 Bauer (21) said no evidence could be found to indicate that heredity, chronic infections, such as tuberculosis and syphilis, an antecedent attack of influenza or pneumonia, or the use of tobacco were factors in causing this neoplasm. Klotz (209) thought it appeared highly probable that chronic irritation of the respiratory tract was of significance and that trauma, bacterial, physical and chemical agents were all incriminated. According to Boland (35) in 1938 there was no more evidence that cancer of the lung is produced by tobacco any more than there was evidence that it is not.

In 1939 Muller (281) concluded that the great increase in tobacco consumption was primarily responsible for the increase in pulmonary cancer of the lung but that the presence of carcinogenic agents besides smoking was also indicated. Also in 1939 Metropolitan Life Insurance Company statisticians (273) said that if smoking were of appreciable etiologic significance the death rate might be expected to increase more rapidly among women than among men but the reverse was the case and at the present (1939) it appeared doubtful that smoking was a factor of etiological importance in cancer of the lung. However, Ochsner and DeBakey (298) said the increase in smoking with the universal custom of inhaling was probably a responsible factor. Assman (14) thought that prolonged chronic stimulation of bronchial mucosa by tobacco smoke could initiate the development of a cancer.

In 1940 Hammond (160) attributed the higher rate of lung cancer in the male sex to the excessive use of tobacco but thought that other irritating elements, such as chemical, mechanical, bacterial, thermal and radioactive agents, might be a contributory factor. Menne and Anderson (271) felt that the increased consumption of tobacco by men, as compared to that by women, was

of great significance in the etiology of lung cancer. In 1940 Macklin and Macklin (255) wrote that the evidence available did not warrant the assumption that primary lung cancer was due to chronic inflammation.

Ochsner and DeBakey (297) in 1941 again expressed their conviction that there was a significant relationship between the increased incidence of cancer of the lung and the increased production of tobacco. Wallace and Jackson (432) related the increase in bronchogenic carcinoma in women in the past several decades to their increased smoking habits. Grace (139) in 1943 said the carcinogenic agent in the tar of tobacco smoke might be the causative factor in some biologically susceptible individuals. In 1946 Ochsner (299) said, "Whereas undoubtedly smoking is not the only cause of lung cancer, it is the most important, and were it not for the constant and continued irritation from prolonged smoking, cancer of the lung would develop in relatively few cases." In 1947 Ochsner *et al.* (300) reversed the decision of Ochsner and DeBakey (297) by stating "both occupation and smoking which have been particularly emphasized by some observers as possible etiologic factors and which we were inclined previously to consider more seriously, were found to have no special significance" in their study of 412 cases. Kennaway and Kennaway (201) said, "The higher mortality from cancer of the lung in towns, the low mortality in agricultural occupations, and the absence of social gradient are compatible with an etiological factor in the air such as coal smoke" but warned that in any comparison of urban and rural areas, the question of facilities for diagnosis must be considered. According to Mandell (256) many types of lung irritants have been suspected and studied from the standpoint of lung cancer etiology, but none could be indicted and convicted.

In 1948 Bradshaw (42) said that there was no experimental or clinical evidence to support the theory that the smoking habits of men were responsible for the greater incidence of lung cancer and Hayden (168) expressed the opinion that there was no proved carcinogenic agent known for bronchogenic carcinoma or cancer elsewhere in the body. Fried (124) did not think the studies of lungs of heavy smokers revealed changes in the bronchi or pulmonary parenchyma that could be blamed on smoking.

"Excessive smoking," according to Wynder and Graham (451), "and in particular cigarette smoking, over a long period, is at least one important factor in the striking increase of bronchiogenic carcinoma." Levin *et al.* (233) reported that their data suggested, but did not establish, a causal relation between cigarette smoking and cancer of the lung but that pipe smokers and cigar smokers had no more cancer of the lung than did nonsmokers. Deibert (80) warned against concluding that the recent stories implicating excessive smoking, especially cigarettes, tell all or most of the story of respiratory carcinogenesis and pointed out that since no other environmental factor was considered the incorrect impression might be produced that smoking apparently represents the only important cause in the production of lung cancer; he felt it advisable not to close our eyes to the possible existence of other carcinogenic agents. In 1954 Hueper (189) concluded that "the existing evidence neither proves nor strongly indicates that tobacco smoke, and especially cigarette smoking, represents a major or even predominating causal factor in the production of cancers of the respiratory tract."

Steiner (404) in 1956 said the problem of the etiology of lung cancer was greatly complicated by the probability of multiple factors. Stocks (409) said that while undoubtedly tobacco smoking was an important factor in the causation of lung cancer, it had not yet proved to be "the cause." According to Russ (363) in 1955, if only one in 100 heavy smokers developed cancer of the lung, the other 99 must have an immunity to the carcinogen that outweighs the susceptibility. In the same year Ornstein and Lercher (308) pointed out that since 1875 irritants had been suspected of leading to changes in the bronchi and so they were not impressed by the present controversy. In 1957 Nasr (286) remarked that the evidence presented so far might point to tobacco as a suspect but could not afford an "incontrovertible proof" of its guilt as a pulmonary carcinogen. In 1956 Lowe (249) reported that in his study the differences in the amount smoked between patients with respiratory tuberculosis and controls were similar to the differences between patients with cancer of the lung and their controls as reported by Doll. The percentage of present cigarette smokers was approximately the same among the white male patients with cancer of the lung, cancer of the larynx, tu-

berculosis, and even appendicitis, in a study made by Rigdon and Kirchoff (342). In 1956 Rosenblatt and Lisa (359) said, "The relationship between the increased incidence and the rise in cigarette consumption is purely speculative. . . . The incidence of lung cancer appears to be related far more to the diagnostic facilities available." Rigdon and Kirchoff (341) showed the incidence of lung cancer to be in direct proportion to the number of doctors and hospital beds per 100,000 population.

In their study of cancer in South Africa, Higginson and Oettle (174) found the incidence of lung cancer in the Bantu male significantly lower than expected in the white and colored male in the United States, but only slightly less than expected from a Danish population and said that if the lung cancer among Bantu males is to be attributed to cigarette smoking certain additional influences must be considered in view of the relatively small average amount smoked. In 1957 Wynder (452) stated that 80 per cent of all lung cancer cases occurring in men would not have resulted without tobacco smoking. In 1960 Davies (78) expressed the opinion that well over half the cases of lung cancer in the United States resulted from cigarette smoking. However, when the percentage of smokers is considered along with the frequency of lung cancer, it is obvious that the ratio is quite small. Based upon the estimate that 80 per cent of men who smoke cigarettes (146) and that one of each 10 smokers will die from lung cancer (78), the predicted number of deaths will exceed the estimate of Cutler and Loveland (76) who predicted in 1955 that, of 1000 white male smokers now 40 years of age, 12 may be expected to develop lung cancer by 60 years of age, 30 by 70 years, and 43 by 80 years.

Doll *et al.* (90) found a distinct relationship in 16 countries between cigarette smoking habits in 1930 and the lung cancer rates of 1952 to 1954. "The principal exceptions are the U.S.A. and Japan, in both of which the mortality is substantially lower than would be expected from their apparent level of cigarette consumption. For the U.S.A. it has, however, often been suggested that the habit of throwing away a large unsmoked butt may contribute to the relatively low mortality." This possibility was also stressed by Kennaway (203) who made some preliminary measurements in England. Factual data for the size of dis-

carded butts on a large scale have been lacking until the recent report by Hammond (163).

Passey (315) pointed out in 1957 that medical authorities agree that cigarette smoking plays a contributing part in the development of lung cancer, but that what has not been publicized is the fact that there is no positive proof that smoking is the primary cause—"just because a man dies of lung cancer and also smoked during his lifetime, it is not justifiable to call smoking the primary cause." Passey suggested that bronchitis may be the primary cause of lung cancer. It is of interest to note that the United States has the highest yearly cigarette consumption per adult male; England's rate is less, yet the English have a lung cancer death rate of 251 per million while the American rate is 112. Dutch adult males consume only one-fourth as many cigarettes as males in the United States, yet the Dutch have a lung cancer death rate of 133. Passey also asked why the lung cancer incidence in the United States is proportionately much higher in the eastern than in the western states; why Wales has a lung cancer rate some 40 times greater than other areas of England although the bronchial cancer incidence has no corresponding increase; why the lung cancer incidence began to rise before the turn of the century and before the time, the World War I era, that cigarette smoking became general.

Smoking and Cancer of the Lung

The hypothesis that smoking causes lung cancer is largely based on studies comparing the smoking habits of lung cancer patients with the smoking habits of patients with other diseases or of healthy people and on studies in which the causes of death are compared in groups of people whose smoking habits have previously been established. Cutler (77) reviewed 14 retrospective studies (44, 91, 216, 234, 261, 274, 281, 365, 370, 372, 435, 439, 451, 453) made between 1939 and 1954 and found that all showed a greater risk of developing lung cancer for smokers than nonsmokers and a still greater risk for heavy smokers. However, the relative risk varied from 1.2 to 36.4 for all smokers and from 1.9 to 79.0 for heavy smokers. In reviewing these studies, it is interesting to note that the percentage of nonsmokers among the lung cancer patients varied from 0.5 (91) to 14.6 (372) and

among the controls from 4.5 (91) to 31.0 (274). The percentage of heavy smokers among the lung cancer patients varied from 18.3 (372) to 86.4 (451) and among the controls from 9.2 (372) to 57.0 (439). Mills and Porter (274) did not give the percentage of heavy smokers in their study; however, they found no significant difference between the cancer victims and the controls in the percentage of cigarette smokers who smoked one pack or more a day. In five studies cigars and pipe tobacco were converted to an equivalent number of cigarettes by considering one cigar equal to five cigarettes and one pipeful of tobacco to two and one-half cigarettes (94, 439, 451, 453), or one ounce of tobbaco a week equal to four cigarettes a day (91), or one cigar equal to 10 cigarettes and one-fourth can of tobacco or less equal to 10 or less cigarettes, one-fourth to one-half can equal to 11 to 20 cigarettes and one-half can or more equal to 21 or more cigarettes (365). Levin (233) said in his study that pipe and cigar smokers had no more lung cancer than nonsmokers.

Mills and Porter (274) said the incidence of lung cancer had a more rapid increase in recent years than all other neoplastic diseases in adult males. Wynder and Graham (451) noted that patients with bronchogenic cancer in their forties and fifties had smoked more heavily than those who developed this cancer at a later age which might indicate that the greater the irritation the sooner cancer will develop in a susceptible person. McConnell *et al.* (261) said that heavy cigarette smoking may be a factor in the etiology of lung cancer. Doll and Hill (91) concluded that the association between cigarette smoking and lung cancer is real; however, they pointed out that it would be a "most unlikely event" that tobacco smoking contributed to the development of all cases. Schrek, *et al.* (372) said the relatively low percentage of deaths of cancer of the respiratory tract as compared to the high percentage of smokers indicated that smoking was at most only a weak carcinogenic agent.

Sadowsky, *et al.* (365) in 1953 warned that retrospective histories of smoking are not likely to give a precise account of actual past smoking habits and, if smoking is cancer-producing, that indulged in just prior to the development of the disease is less important than that indulged in at a more remote time. They concluded "aside from the statistical significance and apparent

realness of these associations there remains the question of whether smoking is etiologically related to lung cancer." In their study an association of about the same degree as that observed with lung cancer was shown between cancer of the larynx and cigarette smoking. Gsell (146) in 1957 found that 95.6 per cent of the cases of laryngeal carcinoma occurred in smokers but pointed out there has been no increase in the frequency of carcinoma of the larynx during the last decade.

Since 1954 six additional retrospective (146, 156, 219, 333, 373, 454) and four prospective (92, 103, 161, 162) studies have been reported. These also show a higher percentage of smokers among the lung cancer patients than among the controls and that the lung cancer risk is greater for heavy smokers.

There is some variation in the different studies in the United States as to the percentage of the population that smokes. Hammond and Horn (162) found that 63.5 per cent of white males between 50 and 59 years of age smoked and 46.1 per cent of those between the ages of 60 and 69 years. Mills and Porter (275) observed that 77 per cent of white males 20 to 29 years of age smoked, 78 per cent of those 30 to 39 years, 80 per cent of those 40 to 49 years, 75 per cent of those 50 to 59 years, 65 per cent of those 60 to 69 years, and 43 per cent of those 70 to 79 years. Kirchoff and Rigdon (205) reported the smoking habits of 6,374 college students in Texas. Of the white male students 15 to 19 years of age, 35.7 per cent smoked, 30.4 per cent smoking one pack of cigarettes a day. Of the white male students 20 to 29 years of age, 54.2 per cent smoked and of these 57.1 per cent smoked a pack of cigarettes a day. In a group of white males 40 to 49 years old 74.7 per cent smoked cigarettes and of these 24.1 per cent had smoked one pack a day, 6.7 per cent one and one-half pack a day, and 5.5 per cent two packs or more a day for 16 years or longer (206).

The pattern of tobacco smoking in the United States, based upon 45,000 persons 18 years of age and over, was published by Haenszel and Shimkin (157). This is the most comprehensive study so far published on this phase of the problem. Of all males over 18 years of age, 59.1 per cent reported that they had used cigarettes regularly at some time. This apparently is a rather low percentage. In a survey made by Kirchoff and Rigdon (206), the

percentage of white male present and past ciagarette smokers 15 years of age and over was 72.1. According to Hammond (163), approximately 60 per cent of the men and 30 per cent of the women in the United States over 18 years of age smoke cigarettes. Doll (93) in 1953 stated that some 95 per cent of men and 42 per cent of women in London would be classified as smokers. Since smoking is such a common habit and lung cancer is relatively infrequent in those who smoke, there must be some as yet unknown factors that enter into this mechanism. Rigdon and Kirchoff (342) observed that 97.2 per cent of the white males who had smoked for 16 years or longer did not have lung cancer according to the medical record at the time of their survey.

Objections to Theory that Smoking is the Cause of Lung Cancer

In 1958 Cornfield, *et al.* (70) pointed out, "The growing and consistent body of evidence has had no noticeable effect upon the viewpoint of a small but important group of individuals who would deny a causal role of cigarette smoking in cancer of the lung. Among these critics are Little (241) and Hartnett (167), spokesmen for the American tobacco industry. Berkson (25, 26) has been critical of many aspects of the statistical studies, and his reservations are, in part, also evident in papers by Neyman (292) and Arkin (12). More general objections by Fisher (119, 120), Greene (143), Hueper (187), Macdonald (251), Rigdon (343), and Rosenblatt (360) have been published."

Many conferences were held during the nineteen-fifties to discuss cancer of the lung (415) and several official bodies in the Netherlands, Sweden, England, the United States and the World Health Organization (50, 269, 270, 289, 399, 401, 448) have supported the theory of the relation of smoking to lung cancer. "So firmly, and even fanatically, is this opinion held in some quarters," cited Berkson (27) in 1959, "that to question it is to court the charge of being biased, surreptitiously a partisan of the interests of the 'tobacco trust,' indecisive where 'action' is wanted, an obstructor of scientific and medical progress, or simply obstreperous. Such attacks are not pleasant to receive, as I know from personal experience, but they are not fatal."

Cornfield, *et al.* (70) discussed recent evidence in the lung cancer-smoking controversy and commented upon some of the

criticism given by other investigators. One important problem discussed in their paper is that referable to inhalation. Lickint (239), Breslow, *et al.* (44) and Schwartz and Denoix (373) found the relative risk of lung cancer to be greater among inhalers than among non-inhalers, while Doll and Hill (91) found no difference between patients with lung cancer who inhaled and those that did not inhale. Cornfield, *et al.* (70), Hueper (187), and Macdonald (251) have emphasized that this observation by Doll and Hill is contradictory to the smoking-lung-cancer relationship. Cornfield, *et al.* (70) said, "It must be admitted that there is no clear explanation of the contradiction posed by the Doll-Hill (91) findings, though a number of plausible hypotheses could be advanced."

This problem of inhalation becomes a basic factor referable to smoking and lung cancer because pipe and cigar smokers usually do not inhale and yet they are included in some studies (91, 94, 365, 439, 451, 453) by converting the number of pipefuls of tobacco and/or cigars into an equivalent number of cigarettes. Based on Sadowsky's (365) method of conversion, a man who smokes three cigars a day would be considered, statistically, a heavy smoker (30 cigarettes a day). Should the same man also smoke 10 cigarettes, he would be classified as a "chain smoker." Some people are old enough today to recall that "self-rolled Bull Durham" cigarettes were popular before the advent of "machine-made" cigarettes. The rate of pyrolysis, and thus the available carcinogen, no doubt would be different in the two types of cigarettes. This factor has not been evaluated in the relationship between cigarette smoking and lung cancer.

Correlations have been made between the recorded deaths from lung cancer and the consumption of tobacco. There has been a progressive increase in the per capita consumption of tax-paid tobacco products in the United States since 1900 (157); however, the authenticity of death certificates for establishing the cause of deaths has been seriously questioned (344, 345, 413). It has been pointed out, "If each one of us (physicians) fails to recognize only one case of cancer of the lung or makes an erroneous certification of death, think what this would mean to our statistics on cancer of the lung" (344). Attempts to compare mortality and tobacco consumption in different countries are hazardous. In the

United States the consumption of cigarettes is much higher than in England. However, the frequency rate of lung cancer is higher in England than in the United States (363). Doll (95), in commenting on this relationship, said, "It appears that the relationship between consumption and subsequent death rates is reasonably close, with the exception that the rate in Britain is too high and the rate in the U.S.A. substantially too low. To a small extent the anomalous position of the United States can be explained by the high proportion of young people in its population. A more important factor, however, may be that smokers in the U.S.A. throw away a larger unsmoked butt than is commonly discarded in Europe."

The explanation given by Hammond (163), a statistician, for the difference in the frequency of lung cancer in the United States and England and Wales is somewhat different from that given above by Doll. "When cancer kills, it is usually widespread in the body at the time of death, and in many cases it is by no means easy to ascertain the site of origin of the disease. These facts suggest the following hypothesis. When cancer is present at the time of death both in the lungs and in other parts of the body and the primary site cannot be determined with accuracy, there is a tendency in England and Wales to record lung as the primary site and a tendency in the United States to record some other organ as the primary site." Data such as this, in our opinion, would be unsatisfactory for scientific evaluation. Data at present, according to Hammond (163), do not support the hypothesis that a substantial part of the difference can be attributed to variations in the amount of cigarette smoking in the two countries. Furthermore, the data at present are insufficient for any definite conclusions to be drawn on why reported lung cancer death rates are so much higher in England and Wales than in the United States (163).

Larson, *et al.* (225) recently discussed the importance in all problems referable to tobacco of an accurate measurement of the amount of tobacco smoke consumed by the individual. "There may be little pharmacological difference between a smoker who deeply inhales the greater part of the smoke of a very few cigarettes, and one who puffs but a small part of the smoke of a very great number, leaving most of his cigarettes smouldering away

on an ash-tray." These authors give a classification of cigarette smokers according to daily consumption, compiled from 50 individual studies and point out that "any quantitative yardstick applied to one smoker is not necessarily valid for another." In 1872 it was pointed out in England that "to all constitutions tobacco is hurtful when used in excess; and to many constitutions it is injurious in any quantity, however small" (268).

In 1959, Doll (96) compared the data on 187,783 men between the ages of 50 and 69 years who died of lung cancer in the United States with the data on 24,354 men 35 years of age and over who died of the same disease in Great Britain and concluded that the association of lung cancer and cigarette smoking is not an artifact resulting from the method of study, but that it does not necessarily follow that smoking causes the disease. He suggested (a) that smoking determines the site of cancer appearance, (b) that the precancerous condition induces smoking, and (c) that smoking and cancer result from a common factor. However, according to Doll, the observations cited against the causal relationship, (d) the increasing disparity in smoking habits, (e) the disproportion between the mortality from lung cancer and the cigarette consumption in different countries, and (f) the conflict over the effect of inhaling, are not sufficient reasons for abandoning the hypothesis of causality.

In June of 1960 the "smoking-lung controversy" was summarized by Berkson (28) as follows: "The investigations exhibiting a statistical relation between smoking and death rates have not provided a definite answer to the problem of lung cancer. On the contrary, they have raised more questions, and more difficult ones, than they were planned to answer. Smokers, in these studies, appear to have larger death rates, not only from cancer of the lung, but from cancer of many other organs, including some remote from the lungs, such as cancer of the prostate, and from all classes of other diseases than cancer, as well. If these statistical results are not spurious—and it is just possible that they are not—and denote an active effect of smoking, they promise to reveal fundamental biologic forces of which we are at present entirely in the dark, including perhaps ones involved with the origin of cancer. This should be reflected in an investigative program to search out their meaning, a program of scope

and intensity adequate to deal effectively with the many complex problems that are involved. Vast funds are available today for cancer research. A large share of them is at the disposal of organizations whose spokesmen have committed them to a primary responsibility vis-a-vis smoking and cancer. If an appreciable portion of these funds is not devoted by these organizations to an elucidation of the problems posed by the statistical studies, it will be a fair interpretation that, however much public notice these studies have afforded them, and however strident are the declarations of concern with the lung-cancer problem, the statistics are not *really* considered significant. Perceptive minds have noted that if one wishes to know what a scientist believes, one should not attend to what he says, but to what investigations he projects." In January 1960 Hammond presented his views at a symposium after which a newspaper reporter asked, "Have you not changed your position?" Actually, he had not changed his position, according to Berkson (28), "but perhaps he has changed his emphasis. He now gives cancer of the lung and smoking only an incidental part in the general effect of 'the air we breathe'."

Risk of Developing Lung Cancer

There is a wide variation in the relative risk of developing lung cancer as shown previously in Cutler's (77) study. Dorn (104) in 1960 stated that the death rate from lung cancer for men who smoked more than a pack of cigarettes per day was 14 times greater than the rate for nonsmokers at ages 55 to 59 years, 19 times greater at ages 60 to 64, and 11 times greater at ages 65 years or more. The death rate for coronary heart disease among regular users of cigarettes is 63 per cent higher than the rate for non-smokers (103). Based upon the mortality experience of nearly 200,000 veterans who died between July, 1954 and December, 1956, Dorn (103) found the death rate for men who used tobacco 32 per cent greater than for nonsmokers. Men who had smoked regularly only cigarettes had the highest death rate of all groups of smokers and 58 per cent greater than the rate for nonsmokers. The rate for ex-cigarette smokers was 31 per cent greater than that for nonsmokers. The excess mortality of regular cigarette smokers was greater for heavy smokers than for light

smokers. The mortality ratio for regular cigarette smokers was 9.85 times that for nonsmokers. The death rate for men smoking more than a pack of cigarettes a day was 16 times that of nonsmokers. Regular cigarette smokers also are subject to an increased risk of dying from cardiovascular disease, from certain respiratory diseases such as bronchitis, pleurisy and emphysema, from ulcers of the stomach and duodenum, and cirrhosis of the liver. Similar findings in a study made in 1954 for the American Cancer Society by Hammond and Horn (162) were the bases for the following comment by Berkson (25), "The question raised by the findings in the American Cancer Society study of higher death rates among cigaret smokers is not 'Does cigaret smoking cause cancer of the lungs?' so much as it is 'What disease does cigaret smoking not cause?' "

Pros and Cons of the Smoking Controversy

Burney (50), the Surgeon General of the United States Public Health Service, in 1959 published an article expressing the position the Public Health Service had taken on the cigarette-lung-cancer controversy. In this the Surgeon General said, "The weight of evidence at present implicates smoking as the principal etiological factor in the increased incidence of lung cancer." It was pointed out by Berkson (29) that this article by the Surgeon General "is characterized by an imprecision of language and thought that renders inaccurate almost every important point it deals with. In particular, it contains some frank errors of omission and commission which are misleading with regard to basic questions at issue. . . . An argument for smoking as a cause of lung cancer is advanced on the basis of the lower mortality from lung cancer among women. . . . Here it is stated that 'nonsmoking women have about the same lung cancer death rate as nonsmoking men,' which, almost certainly, is not true. The death rate for men is greater than for women, not only for cancer of the lung but for cancer of almost every organ that is comparable in the two sexes and for virtually every noncancerous disease also. . . . An even less explicable lapse is Burney's failure to cite the experiments performed in the Public Health Service itself . . . in which administration of cigarette smoke to mice failed to produce lung cancer. The prospective studies show more excess

deaths among the smokers from other causes than lung cancer, and these other causes are distributed among all classes of disease. If the statistical results are not spurious but reflect some real biological effect, this points to a generalized, not a local, effect."

A variation does occur within the Public Health Service in the interpretation of scientific data on smoking and lung cancer. It was pointed out, however, by Rigdon (346) that "this governmental agency should not be criticized because there are differences of opinion among its members, but these facts should be given to the public when these opinions have been arrived at by responsible investigators." As previously stated, the Chief of the Epidemiology Section of the National Cancer Institute (134), which is part of the Public Health Service, pointed out in 1955 that between 1930 and 1938 when the diagnosis of bronchogenic carcinoma appeared on a death certificate it was tabulated with cancer and other malignant tumors of other respiratory organs. It was not until 1939 that deaths which physicians charged to bronchogenic carcinoma were separately identified in official statistics. The Surgeon General also did not discuss the variations in the relative risk for developing lung cancer, from 1.2 to 36.4 for all smokers and from 1.9 to 79.0 for heavy smokers, that Cutler (77), a member of the Public Health Service, found in 14 studies on smoking habits. Furthermore, the Surgeon General did not emphasize what Hammond (163), statistician for the American Cancer Society, said in 1958 about the large and unexplained difference between reported lung cancer death rates in England and Wales and in the United States and about the problem of accuracy of diagnosis which "plagues us whenever we attempt to compare death rates from cancer of specific sites in different periods of time, different countries, or different sections of the same country." Other specific objections to the thesis that cigarette smoking is the primary cause of lung cancer may be found in the following publications: 12, 25, 26, 27, 29, 67, 121, 122, 143, 187, 251, 292, 344, 346, 360.

An editorial (416) published in the Journal of the American Medical Association in 1959 observed that a number of authorities who have examined the evidence on smoking and lung cancer do not agree with those who consider that the evidence to date implicates smoking as the principal etiological factor in the in-

crease in lung cancer: "Although the studies reveal a relationship between smoking and cancer that seems more than coincidental, they do not explain why, even when smoking patterns are the same, case rates are higher among men than among women and among urban than among rural populations. Neither the proponents nor the opponents of the smoking theory have sufficient evidence to warrant the assumption of an all-or-none authoritative position."

Dorn and Baum (105), of the National Institute of Health, in 1955 observed the deaths among cigarette workers were 32 per cent lower than the expected number for all causes, 27 per cent lower for all forms of cancer, 14 per cent lower for cancer of the respiratory system, 33 per cent for cardiovascular disease, and 20 per cent lower for coronary disease. Haag and Hanmer (154) studied the same general population of tobacco workers and found a higher-than-average percentage of smokers and a much-higher-than-average ratio of smoking to be associated with greater longevity, relatively lower death rates from cancer and cardiovascular diseases and respiratory cancer rates no higher than in the general population. The best published report on the amount of tobacco being smoked was based on a survey made by Finkner, *et al.* (118) among the above group of tobacco employees. The techniques used to establish the amount of an individual's smoking emphasizes the uncertainty of the data obtained by others.

Urban and Rural Cancer of the Lung

In support of the theory that smoking is a significant factor in the etiology of lung cancer has been the observation that more cases of lung cancer occur in individuals living in urban areas than among those living in rural areas (64, 75, 135, 178, 201, 220, 335, 336, 410). Stocks (409) in 1955 said that the progressive increase in mortality followed the increasing industrialization, population density, and prevailing direction of smoke drift in "a remarkable way." However, Shik (380) found no more lung cancer among urban than rural populations.

Rigdon and Brindley (347) in 1950 reviewed the data on lung cancer in the senatorial districts of Texas and did not observe any specific environmental factor that suggested a significant

role in the etiology of pulmonary neoplasms. The frequency of cancer of the lung was reviewed in 254 counties in Texas and correlated with (a) the population as recorded in the census of 1950, (b) the number of general hospital beds in 1951, and (c) the number of physicians who were members of the state medical association. From these data the number of deaths from cancer of the lung in each county was related to the number of doctors and the number of hospital beds. Factors of better diagnosis and the distribution of cases of cancer of the lung throughout the states must be carefully evaluated when any specific etiologic agent is proposed (348).

In 1960 Levin, *et al.* (235), in reviewing the problem of lung cancer, said that in 1940 the urban-rural difference in cancer risk based on vital statistics suggested that tumors involving the respiratory system, buccal cavity and esophagus showed a higher urban-rural ratio than tumors in either the stomach or skin. "The gross urban-rural differences in cancer incidence undoubtedly reflect the effect of many factors and in the absence of controlled classification of related population variables the linking of specific urban-rural variations to suspect factors can at best be only suggestive. There is the further problem that the effects for special risk groups are diluted when combined with the remainder of the population not subject to these risks, so one could not expect examination of geographical variation to yield unequivocal leads on factors associated with specific sites" (235). In the United States since 1930 there has been a marked shift in the population from rural to urban areas. Therefore, the location of an individual at the time of death, as shown by vital statistics, will not indicate the environment in which he may have lived for the greater part of his life. Certainly, the interpretation and reconciliation of incidence rates from registry and survey sources may be more complex than previously thought; the problem, according to Levin, *et al.* (235) calls for further investigation.

Kotin (213) in 1956, after reviewing the problem of atmospheric pollution, pointed out, "A basic and almost universal observation has been the demonstration that urban residence carries with it an increased liability to the development of lung cancer. . . . Differences in urban and rural lung cancer mortality figures may be nothing more than another manifestation of the time of

peak incidence. The dose of a carcinogenic agent represents the product of the duration of exposure multiplied by the concentration of the carcinogen. Rural areas differ from urban areas in that pollutants are present in lower concentrations rather than by their complete absence." Doll (97) in 1958 said, "The urban factor seems to have affected the two sexes differently, whereas general atmospheric pollution might be expected to affect them almost equally." Furthermore, the difference between urban and rural mortality is more marked in countries with relatively little pollution (97). It is most interesting that Hammond (163), who is one of the supporters of the theory that smoking is the primary cause of lung cancer, said that although "air pollutants contain carcinogenic substances," it does not "necessarily prove that they cause lung cancer." Kotin (213) said, "There is at present no convincing evidence that tobacco possesses the necessary qualifications for the initiation and promotion of lung cancer."

Frequency of Lung Cancer in the Male and Female

Recently many investigators have thought the preference of lung cancer for the male sex might be a clue to its etiology. However, as early as 1871 Walshe (434) observed that cancer of the lung was more frequent in the male. Actually cancer of the lung is not the only neoplasm with a sex ratio in favor of the male. In 1955 the risk of developing cancer was 60 per cent greater for the male than for the female, exclusive of genital and mammary cancers; the male-female ratio of incidence rates for cancer of the lung and bronchus was 4.49:1, that of the larynx 12.33:1, the lip 7.57:1, the pharynx 5.38:1, the esophagus 4.47:1, and the tongue 3.22:1 (106). Kirchoff and Rigdon (207) reviewed the data on the sex ratio in lung cancer between 1871 and 1957 and found that the male to female ratio in 108 studies (17,609 cases) varied from 0.7:1 to 20.9:1. It would appear from their review that the male-female ratio of lung cancer was approximately 3 or 4 to 1 in 1900 and 5 or 6 to 1 in 1955.

The male-female ratio for lung cancer based upon vital statistics is very different from that obtained from hospital and autopsy data. In Great Britain in 1915 the ratio, based on vital statistics, was 1.5:1, in 1925 it was 1.9:1, and in 1951 it was 5.7:1 (89). In the United States it was 1.7:1 in 1930 and 4.9:1 in 1952 (429). The classification of primary pulmonary neo-

plasms has been changed frequently since 1900. Because of this, certain cases included in broad categories in the early years, today would be classified more specifically. Such factors could contribute to this variation observed in the sex ratio between autopsy series and vital statistics.

Wynder, *et al.* (454) in 1956 pointed out that the changing ratio of male to female lung-cancer cases has been one of the most intriguing clues in this problem of lung cancer. In their study of lung cancer in the female, 16 of the 41 patients with epidermoid cancer were non-smokers. To explain how such could occur, Wynder, *et al.* say it is best to consider an arithmetic example in which there is a population of 10,000,000 adults, half male and half female, in which in any one year lung cancer develops in five out of every 100,000 nonsmokers, whether male or female. Similarly, it is to be supposed that of the smokers, whether male or female, lung cancer develops in 30 out of every 100,000. In such a hypothetical community tobacco is a fundamental factor in the development of lung cancer, whereas sex is not a factor at all. This hypothetical community is further analyzed referable to cancer of the lung in the smokers and non-smokers and finally in this "fairyland of make-believe" Wynder, *et al.* conclude: "In short, in this hypothetical community in which the risk of lung cancer depends on amount smoked and not on sex, the lower frequency of smoking among females results in both a relatively higher incidence of lung cancer among men and a higher percentage of non-smokers among female patients with lung cancer." The prediction of future lung-cancer rates in women, too, must be based on various assumptions. "The fact that even in the youngest age groups half the women are non-smokers and that only five per cent smoke as much as a package a day suggests that although the rate will continue to increase through the years, it is unlikely that it will approach the rate among males. In fact, calculations based on the assumption that 25 years from now a 65 year-old woman will smoke as much as a 40 year-old woman now does suggest that the lung-cancer rate for women is unlikely to increase by more than twofold in the next 25 years on account of tobacco alone." In this same article, Wynder, *et al.* (454) state, "The principles of a scientific demonstration have been so widely misunderstood and disregarded in

the current controversy over smoking and lung cancer that we wish to present a formal outline of the logical steps employed here to go from the basic data to conclusion. . . . The principle of scientific simplicity (Occam's razor) requires that we proceed down this list of categories of hypothesis." Thereupon they summarize the hypotheses for the relationship between smoking and lung cancer.

Racial and Economic Factors Referable to Lung Cancer

Racial differences have been observed in the frequency of lung cancer. Rigdon and Kirchoff (349) noted that cancer of the lung occurred more frequently in the white race than in the Negro. Similar observations were made by Hoffman and Gilliam (178). Duchen (107) in South Africa found the incidence in the Bantu male significantly lower than in the European male. Sitbon and Hadida (386) in Algeria reported no racial variations in the incidence of lung cancer. Steiner, *et al.* (403) observed in California that the incidence of lung cancer among Mexicans was as high in women as in men and that it equalled that in Caucasic men.

In spite of the fact that the rate of smoking tends to increase with income (364), the incidence of lung cancer was more than 40 per cent greater in New Haven, Connecticut among the poor than among other socio-economic classes, according to Cohart (66). Mills and Porter (274) found the incidence greater in the dirtier sections of industrial cities, where pneumonia and tuberculosis rates are also highest; however, they also found cigarette smoking heaviest in the dirty (poor) sections of the city. Cancer of the lung was observed more frequently in the lower economic groups in Denmark (64). Kennaway (204) in 1950 did not discern any influence of social class upon the liability to lung cancer in England. However, Herdan (170) in 1958 did show a change in the social class pattern in lung cancer for men between the years 1921 and 1958.

Histologic Changes in the Respiratory Tract

Hyperplasia, metaplasia and carcinoma-in-situ in the respiratory tract have been observed more frequently in smokers than in nonsmokers (15, 57, 74, 158, 191, 210, 367). These histological

changes have been discussed in their relation to pulmonary neoplasia (33, 74, 294, 330, 367, 433, 442). Similar histologic changes have been observed in pulmonary infections, especially influenza (9, 79, 447). Ide, *et al.* (191) pointed out, "It has not been possible, from the type of evidence obtained, to decide whether the observed changes in the epithelium represent early stages in the development of bronchogenic carcinoma or whether they are simply nonspecific changes associated with the effect of tobacco smoke and air pollutants." The frequency of occurrence of these histological changes in the respiratory tract has varied in different studies. Auerbach, *et al.* (15) observed squamous metaplasia in 1.9 per cent of the slides on patients who had never smoked regularly and 9.5 per cent on the group smoking over a package of cigarettes a day. Carcinoma-in-situ was found in 1.0 per cent of the patients who had never smoked regularly as compared with 6.0 per cent of those who smoked more than a package of cigarettes a day. Knudtson (210) found the bronchial mucosa normal in 50 per cent of the nonsmokers and in 20 per cent of the heavy smokers. These abnormal histologic changes usually were present in the trachea as well as in the larger bronchi (15, 210). It is of interest to observe that carcinoma of the trachea is a rare neoplasm. It has not increased significantly during recent years; however, the mucosal changes, interpreted as "precancerous" in the bronchi, do occur frequently also in the trachea of smokers.

Experimental Studies with Tobacco Smoke

The chemical demonstration of a carcinogen in tobacco condensate and the induction of tumors by local application of it to the skin of mice preceded all attempts to experimentally induce pulmonary neoplasms either by inhalation of cigarette smoke or local application of the condensate to the mucosa of the respiratory tract. The neogenic effect on the respiratory tract has been negative following the inhalation of cigarette smoke in mice (231, 232, 246, 316), rats (148, 316), hamsters (316), rabbits (179), and birds (320). Metaplastic changes occurred in the mucosa of the trachea of dogs following the local application of tobacco condensate (354). Rigdon (350) did not observe any significant morphological changes in either the trachea or the lungs of ducks given tobacco condensate intratracheally. In 1956

Passey (317) said, "During the past three years all those in Great Britain working on the effects of exposure to cigarette smoke or its tars, have been consistent in reporting complete failure to demonstrate any carcinogenic action in a variety of laboratory animals, in spite of the proved presence of traces of the carcinogenic hydrocarbons in cigarette tars." Blacklock (34) injected cigarette tar directly into the hilar region of the lungs of eight rats and obtained one sarcoma and one oat cell carinoma. Campbell (53) and Essenberg (112, 113) obtained tumors in mice, which they thought resulted from the inhalation of cigarette smoke. Leuchtenberger, *et al.* (232) failed to observe tumors in the lungs of mice exposed to cigarette smoke for the greater part of their life span. The successful induction of a tumor in a mouse, however, can of itself have little weight in estimating the carcinogenicity of a substance for man (164).

Spontaneous Pulmonary Tumors in Animals

Spontaneous-occurring pulmonary tumors have been found in the mouse (3, 388, 406, 424, 443). The first of such tumors apparently was reported in 1896 by Livingood (243). Primary lung tumors have been reported in the rat (181, 368), the guinea pig (137, 171), the rabbit (371, 387), fowl (10), the horse (278, 387, 407), bovine (387, 407), the dog (387, 407), the cat (407), the jaguar (387), sheep (115, 407), and the kangaroo (115). It would appear that the pulmonary adenoma of mice represents a specialized situation with limited bearing on the types of tumors that account for the great majority of cases of lung cancer in man (393). The same may be said of all pulmonary tumors in animals; however, it is of interest to know that spontaneous neoplasms do occur in species other than man.

Experimental Production of Lung Cancer

Interest in carcinogenic agents and the experimental production of tumors on the skin of laboratory animals soon was followed by many attempts to induce pulmonary neoplasms with a variety of carcinogens (294, 381, 393, 406). Andervont (4) in 1937 apparently was the first to experimentally induce a squamous cell carcinoma in the lung of a mouse by placing a thread containing 1,2,5,6-dibenzanthracene in the lung through the in-

tact chest wall. Kuschner (223) modified this technique and induced bronchogenic carcinoma in mice and rats by exposure to methylcholanthrene, 1,2,5,6-dibenzanthracene, and 3,4 benzpyrene. Another technique for the production of pulmonary neoplasms has been the implantation of wire mesh pellets impregnated with carcinogenic material (223) and pellets of methylcholanthrene put directly into the lumen of the bronchi (34). Carcinomas have been induced in mice by the tissue transplant technique, a technique in which lung tissue is removed from mouse embryos and implanted along with olive oil solutions of methylcholanthrene or dibenzanthracene into the thigh muscles of adult animals of an inbred strain (394, 395). Pulmonary tumors develop in the lung tissues within the extremities.

Pulmonary neoplasms have occurred in mice (5, 6, 7, 8, 247, 382) and in guinea pigs (171) following the intravenous injection of carcinogenic hydrocarbons. Tumors have occurred in mice (71, 290) and rats (153, 194, 280) following intraperitoneal injection of urethane and in the lungs when urethane was instilled intranasally (377). Adenomas have been demonstrated in the lungs of mice three days after birth when the mothers were given urethane subcutaneously during pregnancy (396).

Since considerable evidence has accumulated to indicate that environmental factors may be significant in lung cancer, inhalation is of particular interest in both experimentally induced pulmonary cancers and in spontaneous-occurring lesions in man. Niskanen (294) in 1949 observed a squamous cell carcinoma in the lung of a rat following intratracheal injection of dibenzanthracene in olive oil. Rigdon (351) produced a variety of neoplasms in the respiratory tract of white Pekin ducks following the intratracheal injection of methylcholanthrene in polysorbate 80 U.S.P. Usually the intratracheal instillation and aerosol exposure of animals to polycyclic hydrocarbons were unsuccessful or the tumor yield was low (223, 327).

The inhalation of 9, 10-dimethyl-1, 2 benzanthracene, although a potent carcinogen for the skin of mice, did not result in any pulmonary tumors (318). Benzpyrene adsorbed upon carbon black was not carcinogenic when given subcutaneously (405) and methylcholanthrene was not carcinogenic when adsorbed on carbon black and given orally to mice (287). The

inactivation of carcinogens by adsorption may be a significant factor in the pathogenesis of pulmonary neoplasms. Carcinogenic substances in the atmosphere, when inhaled, may not be in a biological state such as to produce pulmonary cancers. Kotin and associates (213, 214, 215) have extracted a carcinogen or carcinogens from the atmosphere that induced tumors when applied locally to the skin and pulmonary adenomas when inhaled by mice. Dust collected from the air of cities in England and the United States has produced tumors in the subcutaneous tissue of mice when applied locally (229, 230) and in the lungs when inhaled (263). Campbell (52, 53) was one of the first to observe an increase in pulmonary tumors in mice following the inhalation of dust from tarred roads and the exhaust gases from internal combustion engines.

Inhalation of radioactive particles (55, 129, 418), beryllium (431) and asbestos (296, 391) has resulted in pulmonary neoplasms. An increase in the incidence of lung tumors occurred in strain A mice following long, continued irradiation with gamma rays (248). Several investigators observed the occurrence of pulmonary tumors following the subcutaneous and local application to the skin of carcinogenic agents (284, 369, 436). The intravenous injection of nitrogen mustard (172) and sulfur mustard (173) resulted in an increased number of pulmonary tumors in mice.

Problems in the Diagnosis of Lung Cancer

The problem of the diagnosis of lung cancer was frequently discussed after 1834. By 1930 a marked improvement in the percentage of correct diagnoses had occurred; it was thought to be about 50 per cent correct in the practice of the better physicians, clinics and hospitals (340). Obviously, it would be progressively lower in the practice of those who had less training or inadequate diagnostic facilities. Hruby and Sweany (183) in 1933 considered that 75 per cent of the diagnoses of cancer of the lung should be correct. They stated that a decade before not more than 35 per cent was correctly diagnosed and quoted Ferenzy and Matalosy as reporting 5 per cent correct diagnoses from 1896 to 1900, 28.4 per cent from 1917 to 1925, and 50 per cent in 1925. Pohlen and Emerson (326) in 1942 reviewed 20,000 autopsies performed between 1930 and 1939 in 10 New York general hos-

pitals and found that only 62 per cent of the lung cancer cases had been correctly diagnosed during life. Willis (446) in 1948 stated that carcinoma of the lung was correctly diagnosed during life in only 61 per cent of the cases. In an autopsy study in Moscow for the years 1938 to 1944, 64.5 per cent of the lung cancer cases was correctly diagnosed during life (380).

The difficulties of diagnosis of lung cancer in 1953 were considerable, according to Smithers (397). He pointed out that the accuracy of diagnosis and reliability of death certification must still be under grave suspicion. Even with postmortem examinations mistakes were made. Smithers (397) found four cases in the past few years in which the postmortem examination was in error and three in which the death certificates differed from the postmortem findings. Rigdon and Kirchoff (352) cite a case in which a diagnosis of bronchogenic carcinoma was made from a bronchial biopsy; however, at autopsy the patient had cirrhosis of the liver and a primary hepatic cell carcinoma that had metastasized to the lung and invaded the wall of a medium-size bronchus near the hilus. Kreyberg (221) wrote in 1952, "Up to the present time the general mortality statistics are so unprecise that they must be used with great reservation as a fact for the study of the actual occurrence of lung cancer." In 1957 Shekhter (379) said, "In spite of the undoubtful successful achievements, the clinical and radiological diagnosis of cancer of the lung is still not so satisfactory as it might be."

Treatment

The years from 1930 to 1960 were characterized by the perfection of surgical techniques in the treatment of lung cancer. However, the percentage of cases in which either a cure was obtained or life prolonged probably was insignificant. Graham (140) in 1933 performed the first successful pneumonectomy for a primary bronchogenic carcinoma. The patient, a physician, continued to practice medicine and lived to see Dr. Graham die from a primary bronchogenic carcinoma in 1959 (141). Advances in anesthesiology, blood transfusion, antibiotics and other techniques during these years contributed to a significant decrease in morbidity and mortality associated with lung surgery. Cleland (61) in 1958 made the following comment referable to surgical treatment, "The present status of surgery in the treat-

ment of lung cancer is by no means an entirely happy one. In favourable patients there is a 25 to 30 per cent chance of survival for 5 years but this figure has not advanced significantly in spite of obvious and definite improvements in surgical technique. It seems unlikely that further significant technical improvements will be forthcoming, nor can it be supposed that they will materially improve matters should they come."

Many technical improvements have been made in the pneumonectomy technique used by Graham in 1933 (56, 310, 337). Allison (1) in 1946 recommended the isolation and division of the pulmonary vessels within the pericardium while performing the standard type of pneumonectomy. In 1955 Brock and Whytehead (47) suggested a more radical procedure, including an extensive removal of the mediastinal nodes and the surrounding lung tissue. In 1956 Price Thomas (329) recommended excision of a portion of the main bronchus together with the involved lobe in cases where the lobar bronchus was involved by the malignant growth, a technique known as the "sleeve" resection. A similar technique was suggested for tumors involving the carina and lower trachea (19). Lobectomy was suggested as a palliative procedure in select cases of lung tumors (24, 32). Churchill (59), however, obtained favorable results with lobectomy in selected cases of primary bronchogenic carcinoma. Smith (390) advised a palliative resection in cases of lung cancer to minimize some of the unpleasant terminal symptoms.

Since 1930 there has been an increase in the use of exploratory thoracotomy, with a wide variation in the frequency in which a subsequent pneumonectomy was performed. Rosemond, *et al.* (358) in 1950 performed pneumonectomies upon about 55 per cent of those who were thoracotomized. Before 1945 Lindskog and Bloomer (242) performed pneumonectomies on 38 per cent of their cases and between 1945 and 1948 on approximately 50 per cent. In 1954 Farber (114) said that 50 per cent could be taken as a representative mean. However, in at least 40 per cent of the patients receiving pneumonectomy, there was no evidence of a material increase in the length of life.

The demonstration of the lymph drainage of the lungs by Rouviere (362) in 1932 contributed greatly to both the surgical diagnosis and the treatment of lung cancer. It was shown by

Rouviere that tumors in the right lung spread to the right paratracheal nodes and then upward to the supraclavicular lymph nodes. Tumors from the left lower lobe and the lower portions of the left upper lobe spread to the carinal nodes at the bifurcation of the trachea and then across to the right paratracheal nodes and upward to the right supraclavicular nodes, while tumors in the apex of the left upper lobe of the lung spread upward to the left supraclavicular nodes. Metastasis from the left upper lobe may spread (a) superiorly to the left paratracheal chain to the left supraclavicular nodes and (b) down to the carinal nodes (68, 165, 182, 295, 374, 426). The demonstration of metastatic tumor in the scalene fat pad in the supraclavicular spaces usually was interpreted as an inoperative pulmonary lesion. In the surgical removal of the lung, Healey and Gibbon (169) suggested the intrapericardial ligation of the veins at the atrium when either the tumor or the lymphatic extension surrounded the blood vessels. Early ligation of the pulmonary veins may lessen the chance of blood borne dissemination of tumor cells (17). The extent of mediastinal lymph node involvement with crossing of the lymphatic drainage from one side of the mediastinum to the other was emphasized (51).

The mortality and survival rates following surgery for bronchogenic carcinoma vary (16, 20, 32, 43, 59, 132, 133, 142, 259, 301, 302, 303, 311, 337, 378, 417). At the time of the introduction of total pneumonectomy the operative mortality was around 50 per cent (114). In 1958 the operative mortality for all types of resection, according to Cleland (61), was approximately 10 per cent. The mortality was higher after pneumonectomy than following lobectomy (32, 59, 133, 378). It increased with age (32, 133, 301). Survival was shown to be related to the histologic type of tumor (38, 133, 208, 262, 277).

Aggressive treatment of lung tumors with radiation before 1930 with techniques then available was rarely feasible. However, since 1940 radiological advances have made possible more effective treatment. Radiation therapy has been considered by some to prolong the life of individuals with lung cancer (73, 227, 328, 389, 419). Overholt (312) believed that radiotherapy shortened the life of patients with this neoplasm.

The supervoltage van de Graaff generator introduced about

1950 (422, 444) for treatment of cancer proved to be palliative and not curative in cases of lung cancer (128, 152, 437). In 1950 Marks (257) renewed interest in this country in the technique of "fractionation of the x-ray dosage in space" first advocated by Kohler in 1909. This technique was considered more advantageous in the treatment of lung cancer (166, 258).

Whether types of radiation other than photons (x-rays) will represent an advance in our ability to help patients with carcinoma of the lung is far from clear. In 1956, according to Nickson and Roswitt (293), there was no theoretical reason to anticipate a sharp biological difference in response from that already seen with photons. Whether the electron beam can be utilized to great advantage on patients with carcinoma of the bronchus is not yet clear. It is unlikely that the electron beam will come to play a major role in the management of this neoplasm (293).

In 1934 Edwards (110) suggested the use of gamma radiation in the treatment of bronchial tumors. At first radon seeds were introduced into the tumor through the bronchus in tubular containers and later the radon seed was injected into the tumor at the time of open thoracotomy (305, 306, 307). An implant method using gold grains was introduced by Hodt, Sinclair and Smithers (176) in 1952 and subsequently was used in the treatment of bronchial carcinoma.

A new method for the treatment of lung cancer by means of artificial radioactivity was suggested by the studies of Muller and Rossier (282, 283) in 1947. This was based upon the selective fixation following an intravenous injection of a short-lived radioactive isotope. Subsequent observations have shown that such isotopes may be injected by catheter into the pulmonary artery or given directly into the tumor (23). Smithers (398) in 1958, discussing isotopes in the treatment of bronchogenic carcinoma said, "Several new radioactive isotope treatment methods are being tried but most of them offer little prospect of success."

Chemotherapy in bronchogenic carcinoma was influenced by the study of Gilman and Philips (136) and the statement by Rhoads (338) in 1946 that a "limited but favorable experience with the methyl-bis compound in inducing temporary symptomatic remissions in anaplastic carcinoma of the lung has encouraged further trial in this type of disease." Others have

demonstrated the clinical effect of the chloroethylamines on bronchogenic carcinoma (41).

The efficiency of all forms of therapy for lung cancer in 1960 was stated by Jacobs, *et al.* (192) as follows, "It is obvious from the study that present methods of treating pulmonary carcinoma are grossly inadequate. One can hope for better results only from some radically new method of approach. While chemotherapy has not yet introduced much change in this situation we may not be using the optimum agent."

Because of the increased incidence of lung cancer and the low curability rate, a concerted effort began about 1940 to discover the earliest cases for treatment. This was done by physicians advocating frequent physical examinations and by radiographic screening of large groups of individuals (149). In discussing the technique of mass surveys, Guiss (150) in 1960 said, "Mass roentgenographic surveys are probably not the answer because of their expense and lack of selectivity." Furthermore, if chest roentgenograms are to be used efficiently and successfully for the purpose of detecting lung cancer or any other pulmonary disease, scientific criteria must be developed and generally applied to this procedure (151).

Consideration of Smoking and Lung Cancer by Legislative Bodies

Obviously from this review it is evident that the etiology and the pathogenesis of pulmonary cancer in man is not known. Events of the past few years, however, would indicate that this problem is being discussed by legislative bodies both in this country and Europe (67, 269). The alleged effect of smoking on health was brought up for discussion in the House of Commons (269) in 1954 and by a committee of the House of Representatives in the United States (67) in 1957. The latter report (67) gives an excellent summary of the problem.

In April of 1960 a federal court in Pittsburgh, Pennsylvania had the first trial in which a tobacco company was sued for more than a million dollars by a man who alleged he developed a pulmonary cancer from smoking a specific brand of cigarettes. The Court ruled in favor of the tobacco company. The second trial of a similar case was heard in July 1960 in Miami, Florida. In this case the jury was instructed by the judge to consider the following four points: (a) Did the plaintiff (Green) have cancer

originating in his left lung? The answer was yes. (b) Was this cancer the cause or one of the causes of his death? The answer was yes. (c) Was the smoking of Lucky Strikes (cigarettes) a proximate, or one of the proximate, causes of cancer? The answer was yes. (d) Could the American Tobacco Company on or before February 1, 1956 (Green died in February of 1958) by the reasonable application of human skill and foresight, have known that users of Lucky Strike cigarettes, such as Green, would be endangered . . . of contracting cancer of the lung? To this the jury answered no (420). A court official interpreted the ruling as indicating that the jury felt that not enough had been known about the dangers of smoking prior to 1956, when the man's illness was diagnosed, to hold the company financially liable (375).

The jury in the latter case, after hearing testimony for two weeks, decided a medical problem that scientists have been unable to answer in a hundred years. Furthermore, this jury was able to conclude, not only what the etiology of a specific pulmonary tumor was, but also that a primary pulmonary cancer was present in the left apex of the lung when there was no autopsy or thoracotomy and radiological findings were consistent with either an inflammatory or neoplastic lesion. The only neoplasm demonstrated histologically in this case was a biopsy of a supraclavicular lymph node on the right side that showed a squamous cell carcinoma. There was no evidence presented to the Court to show any abnormalities to suggest metastases in any organ or bone.

The legal findings in the above cited cases would suggest that monetary interest in cancer of the lung in 1960 is competing with science in establishing the etiology of this particular neoplasm.

REFERENCES

1. Allison, P. R.: Intrapericardial approach to the lung root in the treatment of bronchial carcinoma by dissection pneumonectomy. *J. Thorac. Surg.*, 15: 99–117, 1946.
2. Amador W., Elias: Carcinoma broncogenico en gente joven. *La Pren. Med. Mex.*, 1955, 20: #9, 189–193.
3. Andervont, Howard B.: Biological studies on the mammary tumor inciter in mice. *Ann. N. Y. Acad. Sci.*, 54: 1004–1011, 1952.
4. Andervont, H. B.: Pulmonary tumors in mice. IV. Lung tumors induced by subcutaneous injections of 1:2:5:6-dibenzanthracene in different media and by its direct contact with lung tissues. *Pub. Health Rep.*, 52: 1584–1589, 1937.
5. Andervont, H. B., and Egon Lorenz: Dibenzanthracene tumors in mice. The production of subcutaneous pulmonary and liver tumors by serum dispersions and lard solutions. *Pub. Health Rep.*, 52: 637–647, 1937.
6. Andervont, H. B.: Dibenzanthracene tumors in mice. The production of subcutaneous and pulmonary tumors by 1:2:5:6-dibenzanthracene adsorbed on charcoal. *Pub. Health Rep.*, 52: 1931–1940, 1937.
7. Andervont, H. B.: Pulmonary tumors in mice. VI. Time of appearance of tumors induced in strain A mice following injection of 1:2:5:6-dibenzanthracene or 20-methylcholanthrene. *Pub. Health Rep.*, 54: 1512–1533, 1939.

8. Andervont, Howard B., and Michael B. Shimkin: Biologic testing of carcinogens. II. Pulmonary-tumor-induction technique. *J. Nat'l Cancer Inst.*, 1: 225–239, 1940.
9. Andiral, Milorad: Tracheal and bronchial squamous metaplasia in influenza. *Arch. Path.*, 68: 104–112, 1959.
10. Apperly, Frank L.: Primary carcinoma of the lung in the domestic fowl. *Am. J. Cancer*, 23: 556–557, 1935.
11. Arkin, Aaron and David H. Wagner: Primary carcinoma of the lung. A diagnostic study of one hundred and thirty-five cases in four years. *J. A. M. A.*, 106: 587–591, 1936.
12. Arkin, Herbert: Relationship between human smoking habits and death rates. An analysis of the American Cancer Society Survey. *Current Med. Dig.*, 22: 37–44, 1955.
13. Ask-Upmark, Erik: Bronchial carcinoma in printing workers. *Dis. Chest*, 27: 427–435, 1955.
14. Assman, H.: Ueber Nikotinschäden: *Munch. Med. Wchnschr.*, 86: 457–460, 1939.
15. Auerbach, Oscar, J. Brewster Gere, Jerome B. Forman, Thomas G. Petrick, Harold J. Smolin, Gerald E. Muehsam, Dicran Y. Kassouny, and Arthur Purdy Stout: Changes in the bronchial epithelium in relation to smoking and cancer of the lung. *New Eng. J. Med.*, 256: 97–104, 1957.
16. Aufses, Arthur H.: Primary carcinoma of the lung. A fourteen year survey. *J. Mt. Sinai Hosp.*, 20: 212–228, 1953.
17. Aylwin, John H.: Avoidable vascular spread in resection of bronchial carcinoma. *Thorax*, 6: 250–267, 1951.
18. Baetjer, Anna M.: Pulmonary carcinoma in chromate workers. *Arch. Ind. Hyg.*, 2: 487–516, 1950.
19. Barclay, R. S., N. McSwain, and T. M. Welsh: Tracheal reconstruction without the use of grafts. *Thorax*, 12: 177–180, 1957.
20. Barthel, Heinz: Ergebnisse nach Lungenresektion beim Bronchialkarzinom. *Thoraxchir.*, 1: 337–341, 1953.
21. Bauer, John T.: A review of the primary carcinoma of the lungs and pleura occurring in 6,000 consecutive necropsies. *Bull. Ayer Clin. Lab. Penn. Hosp.*, 3: 139–188, 1938.
22. Bayle, G. H.: *Réchèrches sur la Phtisie Pulmonaire*. Gabon, Paris, 1810.
23. Becker, Josef, Karl Werner, Helmut Kuttig, Kurt Ernst Scheer, and Günter Weitzel: Das Bronchus-Karzinom in strahlenklinischer Sicht. II. Teil. *Strahlenlherapie*, 103: 348–367, 1957.
24. Belcher, J. R.: Lobectomy for bronchial carinoma. *Lancet*, 1: 349–352, 1956.
25. Berkson, Joseph: The statistical study of association between smoking and lung cancer. *Proc. Staff Meet. Mayo Clin.*, 30: 319–348, 1955.
26. Berkson, Joseph: Smoking and lung cancer; some observations on two recent reports. *J. Am. Stat. Assn.*, 53: 28–38, 1958.
27. Berkson, Joseph: The statistical investigation of smoking and cancer of the lung. *Proc. Staff Meet. Mayo Clin.*, 34: 206–224, 1959.
28. Berkson, Joseph: Smoking and cancer of the lung. *Proc. Staff Meet. Mayo Clin.*, 35: 367–385, 1960.
29. Berkson, Joseph: Statistics and tobacco. Letter to the editor. *J. A. M. A.*, 172: 967–969, 1960.
30. Bidstrup, P. Lesley, and R. A. M. Case: Carcinoma of the lung in workmen in the bichromates-producing industry in Great Britain. *Brit. J. Ind. Med.*, 13: 260–264, 1956.
31. Bignall, J. R.: Editor. Carcinoma of the Lung. Vol. I., *Monographs on Neoplastic Disease*. Gen. ed., D. W. Smithers. E. & S. Livingstone Ltd., Edinburgh and London, 1958.
32. Bignall, J. R., and A. J. Moon: Survival after lung resection for bronchial carcinoma. *Thorax*, 10: 183–190, 1955.
33. Black, Harrison, and Lauren V. Ackerman: The importance of epidermoid carcinoma *in situ* in the histogenesis of carcinoma of the lung. *Ann. Surg.*, 136: 44–55, 1952.
34. Blacklock, J. W. S.: The production of lung tumours in rats by 3:4 benzpyrene, methylcholanthrene, and the condensate from cigarette smoke. *Brit. J. Cancer*, 11: 181–191, 1957.
35. Boland, E. R.: "Stinking Smoke"—A digression. *Guy's Hosp. Gaz.*, 52: 225–232, 1938.
36. Bonsor, Georgiana M.: The incidence of intrathoracic tumours in Leeds. *J. Hyg.*, 34: 218–234, 1934.
37. Bonsor, Georgiana M., and Gretta M. Thomas: An investigation of the validity of death certification of cancer of the lung in Leeds. *Brit. J. Cancer*, 13: 1–12, 1959.
38. Borrie, John: Primary carcinoma of the bronchus: prognosis following surgical resection. (A clinico-pathological study of 200 patients.) *Ann. Roy. Coll. Surg. Eng.*, 10: 165–186, 1952.
39. Boycott, A. E.: Cancer of the lung. *Lancet*, 2: 959, 1932.
40. Boyd, William: Notes on the pathology of primary carcinoma of the lung. *Can. M. A. J.*, 23: 210–217, 1930.
41. Boyland, E., J. W. Clegg, P. C. Koller, E. Rhoden, and O. H. Warwick: The effects of chloroethylamines on tumours with special reference to bronchogenic carcinoma. *Brit. J. Cancer*, 2: 17–29, 1948.
42. Bradshaw, Howard H.: Primary cancer of the lung. *N. C. Med. J.*, 9: 186–189, 1948.
43. Brea, Mario M. J.: Primary carcinoma of the lung. Results of surgical treatment. *Surg.*, 35: 167–173, 1954.
44. Breslow, Lester, LeMar Hoaglin, Gladys Rasmussen, and Herbert K. Abrams: Occupations and cigarette smoking as factors in lung cancer. *Am. J. Pub. Health*, 44: 171–181, 1954.
45. Brines, Osborn Allen, and John Carl Kenning: Bronchiogenic carcinoma. *Am. J. Clin. Path.*, 7: 120–133, 1937.
46. Brinton, Hugh P., Elizabeth S. Frasier, and A. Link Koven: Morbidity and mortality experience among chromate workers. *Pub. Health Rep.*, 67: 835–847, 1952.
47. Brock, Sir Russell, and L. L. Whytehead: Radical pneumonectomy for bronchial carcinoma. *Brit. J. Surg.*, 43: 8–24, 1955.
48. Brockbank, William: The occupational incidence of primary lung cancer. *Quart J. Med.*, N. S. 1: 31–40, 1932.

49. Bruen, Edward T.: Cancer of the Lungs. *A System of Practical Medicine*. Vol. III. p. 460, Ed., William Pepper. Lea Brothers and Co., Philadelphia, 1885.
50. Burney, Leroy E.: Smoking and lung cancer. A statement of the Public Health Service. *J. A. M. A.*, 171: 1829–1837, 1959.
51. Cahan, William G., William L. Watson, and John L. Pool: Radical pneumonectomy. *J. Thorac. Surg.*, 22: 449–473, 1951.
52. Campbell, J. Argyll: Lung tumours in mice and man. *Brit. Med. J.*, 1: 179–183, 1943.
53. Campbell, J. Argyll: The effects of exhaust gases from internal combustion engines and of tobacco smoke upon mice, with special references to incidence of tumours of the lung. *Brit. J. Exper. Path.*, 17: 146–158, 1936.
54. Cancer Morbidity Series. Federal Security Agency. U.S. Public Health Service, 1–10, 1950–52.
55. Cember, H., and J. A. Watson: Bronchogenic carcinoma from radioactive barium sulfate. *Arch. Ind. Health*, 17: 230–235, 1958.
56. Chamberlain, J. Maxwell, and Charles F. Daniels: Surgical Therapy. Chap. X, p. 227 in *Cancer of the Lung*. Milton R. Rosenblatt and James R. Lisa. Oxford Univ. Press, New York, 1956.
57. Chang, Suk C.: Microscopic properties of whole mounts and sections of human bronchial epithelium of smokers and nonsmokers. *Cancer*, 10: 1246–1262, 1957.
58. Chief Inspector of Factories. Annual Report of the Chief Inspector of Factories for the Year 1950, p. 145. *H. M. S. O.*, London. (Reference from Doll (85).)
59. Churchill, Edward D., Richard H. Sweet, Lamar Soutter, and J. Gordon Scannell: The surgical management of carcinoma of the lung. A study of the cases treated at the Massachusetts General Hospital from 1930 to 1950. *J. Thorac. Surg.*, 20: 349–365, 1950.
60. Cleland, J. B.: The pathological lesions in 5,000 Australian autopsies. *M. J. Australia*, 1: 625–631, 1945.
61. Cleland, W. P.: Surgical Treatment. Chap. 21 in Carcinoma of the Lung. Vol. I. Ed., J. R. Bignall. *Monographs on Neoplastic Disease*. Gen. ed., D. W. Smithers. E. and S. Livingstone, Ltd., Edinburgh and London, 1958.
62. Clemmesen, J., and T. Busk: On the apparent increase in the incidence of lung cancer in Denmark, 1931–1945. *Brit. J. Cancer*, 1: 235–259, 1947.
63. Clemmesen, Johannes, Arne Nielson, and Emmerik Jensen: Mortality and incidence of cancer of the lung in Denmark and some other countries. *Acta Unio Internat. Contra Cancrum*, 9: 603–636, 1953.
64. Clemmesen, J., A. Nielsen, E. Jensen: The increase in incidence of carcinoma of the lung in Denmark, 1931–1950. *Brit. J. Cancer*, 7: 1–9, 1953.
65. Clough, Paul W.: The relation of cigarette smoking to bronchogenic carcinoma. *Ann. Int. Med.*, 46: 187–196, 1957.
66. Cohart, Edward M.: Socioeconomic distribution of cancer of the lung in New Haven. *Cancer*, 8: 1126–1129, 1955.
67. Congressional Hearings. False and Misleading Advertising. Hearings before a Subcommittee of the Committee on Government Operations. House of Representatives. Eighty-fifth Congress, July 18–26, 1957. U. S. Government Printing Office, Washington, D.C.: 1957.
68. Connar, Richard G.: Prescalene and deep cervical lymph node biopsy. *Surg. Gyn. Obst.*, 101: 733–743, 1955.
69. Cooray, G. H.: Observations on malignant disease in Ceylon based on a study of two thousand two hundred and ninety-five biopsies of malignant tumours. *Indian J. Med. Res.*, 32: 71–91, 1944.
70. Cornfield, Jerome, William Haenszel, Cuyler Hammond, Abraham M. Lilienfeld, Michael B. Shimkin, and Ernst L. Wynder: Smoking and lung cancer: recent evidence and a discussion of some questions. *J. Nat'l Cancer Inst.*, 22: 173–203, 1959.
71. Cowen, P. N.: Some studies on the action of urethane on mice. *Brit. J. Cancer*, 1: 401–405, 1947.
72. Craver, Lloyd F.: Etiology of cancer. A partial review. *J. A. M. A.*, 105: 1820–1824, 1935.
73. Craver, Lloyd F.: Bronchiogenic carcinoma. *Am. J. Roentgenol.*, 43: 469–478, 1940.
74. Cunningham, G. J., and D. P. Winstanley: Hyperplasia and metaplasia in the bronchial epithelium. *Ann. Roy. Coll. Surg. Eng.*, 24: 323–330, 1959.
75. Curwen, M. P., E. L. Kennaway, and N. M. Kennaway: The incidence of cancer of the lung and larynx in urban and rural districts. *Brit. J. Cancer*, 8: 181–198, 1954.
76. Cutler, Sidney J., and Donald B. Loveland: The risk of developing lung cancer and its relationship to smoking. *J. Nat'l Cancer Inst.*, 15: 201–211, 1955.
77. Cutler, Sidney J.: A review of the statistical evidence on the association between smoking and lung cancer. *J. Am. Stat. Assn.*, 50: 267–282, 1955.
78. Davies, Dean F.: A statement on lung cancer. *CA Bull. Cancer Progress*, 9: #6, 207–208, 1959.
79. DeBaan, P.: Pulmonary changes in Asian influenza. *Ned. Tijdschr. Geneesk.*, 103: 721–726, 1959.
80. Deibert, Austin V.: Cancer of the lung. *J.A.M.A.*, 144: 64, 1950.
81. Demel, Venceslao Cesaris: Il Cancro Primitivo del Polmone. *"Universitas" Societa Editrice*, Roma, 1940.
82. DeMuylder, Ch.: Une opinion belge sur la frequence et l'etiologie due cancer primitif des bronches. *Acta unio Internat. Contra Cancrum*, 9: 562–572, 1953.
83. Denk, W.: Observations on bronchial carcinoma. *Bruns' Beit. zur klin. Chir.*, 186: (3), 282–290, 1953.
84. Denoix, P. F.: Les cancers bronchopulmonaires en France entre 1946 et 1951. *Acta Unio Internat.* Contra Cancrum, 9: 573–579, 1953.
85. Doll, Richard: Specific Industrial Causes. Chap. V in Carcinoma of the Lung. Ed., J. R. Bignall. Vol. I. *Monographs on Neoplastic Diseases*. Gen. ed., D. W. Smithers. E. & S. Livingstone, Ltd., Edinburgh and London, 1958.
86. Doll, Richard: Cancer of the lung and nose in nickel workers. *Brit. J. Ind. Med.*, 15: 217–223, 1958.
87. Doll, Richard: The causes of death among

gas-workers with special reference to cancer of the lungs. *Brit. J. Ind. Med.*, 9: 180–185, 1952.

88. Doll, Richard: Mortality from lung cancer in asbestos workers. *Brit. J. Ind. Med.*, 12: 81–86, 1955.
89. Doll, Richard: Bronchial carcinoma; incidence and aetiology. *Brit. Med. J.*, 2: 521–527, 1953.
90. Doll, R., A. Bradford Hill, P. G. Gray, and E. A. Parr: Lung cancer mortality and the length of cigarette ends. An international comparison. *Brit. Med. J.*, 1: 322–325, 1959.
91. Doll, Richard, and A. Bradford Hill: A study of the aetiology of carcinoma of the lung. *Brit. Med. J.*, 2: 1271–1286, 1952.
92. Doll, Richard, and A. Bradford Hill: Lung cancer and other causes of death in relation to smoking. A second report on the mortality of British doctors. *Brit. Med. J.*, 2: 1071–1081, 1956.
93. Doll, Richard: Smoking and carcinoma of the lung. *Unio Internat. Contra Cancrum Acta*, 9: 495–506, 1953.
94. Doll, Richard, and A. Bradford Hill: Smoking and carcinoma of the lung. *Brit. Med. J.*, 2: 739–748, 1950.
95. Doll, Richard: The Smoking of Tobacco. Chap. VI in Carcinoma of the Lung. Ed., J. R. Bignall. Vol. I. *Monographs on Neoplastic Diseases*. Gen. ed., D. W. Smithers. E. & S. Livingstone, Ltd., Edinburgh and London, 1958.
96. Doll, R.: Lung cancer and cigarette smoking. *Acta Unio Internat. Contra Cancrum*, 15: 417–423, 1959. *Abst., J. A. M. A.*, 170: 1871–1872, 1959.
97. Doll, Richard: Atmospheric Pollution. Chap. VII in Carcinoma of the Lung. Ed., J. R. Bignall. Vol. I. *Monographs on Neoplastic Diseases*. Gen. ed., D. W. Smithers. E. & S. Livingstone, Ltd., Edinburgh and London, 1958.
98. Dorn, Harold F.: The incidence and prevalence of cancer of the lung. *Weekly Pub. Health Rep.*, 58: 1265–1271, 1943.
99. Dorn, Harold F.: Morbidity and mortality from cancer of the lung in the United States. *Acta Unio Internat. Contra Cancrum*, 9: 552–561, 1953.
100. Dorn, Harold F.: The increase in cancer of the lung. *Ind. Med. and Surg.*, 23: 253–257, 1954.
101. Dorn, Harold F.: The relationship of cancer of the lung and the use of tobacco. *Am. Stat.*, 8: 7–13, 1954.
102. Dorn, Harold F., and Joseph I. Horn: The reliability of certificates of deaths from cancer. *Am. J. Hyg.*, 34: 12–23, 1941.
103. Dorn, Harold F.: Tobacco consumption and mortality from cancer and other diseases. *Pub. Health Rep.*, 74: 581–593, 1959.
104. Dorn, Harold F.: Note on cigarette smoking and lung cancer. *Pub. Health Rep.*, 75: 582, 1960.
105. Dorn Harold F., and William S. Baum: Mortality among workers in cigarette factories. *Ind. Med. and Surg.*, 24: 239–241, 1955.
106. Dorn, Harold F., and Sidney J. Cutler: Morbidity from cancer in the United States. Part I. Variation in incidence by age, sex, race, marital status and geographic region. *Public Health Monograph* #29, Public Health Service Publication 418, 1955.
107. Duchen, L. W.: Bronchogenic carcinoma. Incidence and pathology as seen on the Witwatersrand. *S. African J. Med. Sci.*, 19: 65–74, 1954.
108. Dungal, Niels: Lung carcinoma in Iceland. *Lancet*, 2: 245–247, 1950.
109. Editorial. Cancer of the lung. *J.A.M.A.*, 108: 1716–1717, 1937.
110. Edwards, A. Tudor: Malignant disease of the lung. *J. Thorac. Surg.*, 4: 107–124, 1934.
111. El-Gazayerli, Munir: Note on the incidence of intrathoracic tumours in Edinburgh Royal Infirmary. *J. Hyg.*, 36: 449–451, 1936.
112. Essenberg, J. M.: Cigarette smoke and the incidence of primary neoplasms of the lung in the Albino mouse. *Sci.*, 116: 561–562, 1952.
113. Essenberg, J. M.: Further study of tumor formation in the lungs of the Albino mice. *West. J. Surg.*, 65: 161–163, 1957.
114. Farber, Seymour M.: *Lung Cancer*. Charles C Thomas, Springfield Ill., pp. 106–107, 1954.
115. Feldman, W. H.: *Neoplasms of Domesticated Animals*. W. B. Saunders Co., Philadelphia, 1932.
116. Ferrari, Enrico: Tabakrauch und Lungenkarzinom. *Munch. Med. Wchnschr.*, 80: 942, 1933.
117. Filippi, Jose De: El Cancer del Pulmon. Contribucion a su Estudio y Tratamiento. Editorial *"El Ateneo,"* Buenos Aires, 1938.
118. Finkner, A. L., D. G. Horvitz, G. T. Foradori, Jack Fleischer, and John Monroe: An investigation on the measurement of current smoking by individuals. *Univ. N. C. Inst. Stat.*, Mimeo series 177, Chapel Hill, N. C., 1957.
119. Fisher, Ronald A.: Dangers of cigarette-smoking. *Brit. Med. J.*, 2: 297–298, 1957.
120. Fisher, Sir Ronald: Cigarettes, Cancer and Statistics. *Centennial Rev. Arts and Sci.*, Mich. St. Univ., 2: #2, 151–166, 1958.
121. Fisher, Ronald A.: Lung cancer and cigarettes? *Nature*, 182: 108, 1958.
122. Fisher, Sir Ronald A.: Smoking—The Cancer Controversy. *Some Attempts to Assess the Evidence*. Oliver and Boyd, Edinburgh and London, 1959.
123. Fleckseder, Rudolf. Ueber den bronchialkrebs und einige seiner enstehungsbedingungen. *Munch. Med. Wchnschr.*, 83: 1585–1588, 1936.
124. Fried, B. M.: *Bronchiogenic Carcinoma and Adenoma*. The Williams & Wilkins Company, Baltimore, 1948.
125. Fried, B. M.: Primary carcinoma of the lungs. Further study, with particular attention to incidence, diagnoses, and metastases to the central nervous system. *Arch. Int. Med.*, 40: 340–363, 1927.
126. Frissell, Lewis Fox, and Leila Charlton Knox: Primary carcinoma of the lung. *Am. J. Cancer*, 30: 219–288, 1937.
127. Fruhling, Louis et Horrenberger, David: De l'augmentation actuelle de la frequence des cancers pulmonaires d'apres la statistique necropsique. *Bull. L'Assn. fran. L'Etude du Cancer*, 39: 17–30, 1952.
128. Garland, L. H., and M. A. Sisson: The

results of radiotherapy of bronchial cancer. *Radiol.*, 67: 48–62, 1956.
129. Gates, Olive, and Shields Warren: The production of bronchial carcinomas in mice. *Am. J. Path.*, 36: 653–671, 1960.
130. Gelfand, Michael: Malignancy in the African. *S. African M. J.*, 23: 1010–1016, 1949.
131. Gharpure, P. V.: Incidence of primary carcinoma of the liver and other organs as inferred from autopsy work, 1926 to 1946. *Indian Med. Gaz.*, 83: 5–6, 1948.
132. Gibbon, John H., Frank F. Allbritten, John Y. Templeton, III, and Thomas F. Nealon: Cancer of the lung. An analysis of 532 consecutive cases. *Ann. Surg.*, 138: 489–501, 1953.
133. Gifford, John Hamilton, and J. K. B. Waddington: Review of 464 cases of carcinoma of lung treated by resection. *Brit. Med. J.*, 1: 723–730, 1957.
134. Gilliam, Alexander G.: Some aspects of the lung cancer problem. *Mil. Med.*, 116: 163–174, 1955.
135. Gilliam, Alexander G.: Mortality attributed to lung cancer in the large cities of the United States in 1948 and 1949. *J. Nat'l Cancer Inst.*, 15: 1307–1312, 1955.
136. Gilman, Alfred, and Frederick S. Philips: The biological actions and therapeutic applications of the B-chloroethyl amines and sulfides. *Sci.*, 103: 409–415, 1946.
137. Goldberg, S. A.: The occurrence of epithelial tumors in the domesticated animals. *J. A. V. M. A.*, 58: 47–63, 1920.
138. Goldblatt, M. W.: Occupational cancer. *Practitioner*, 181: 151–163, 1958.
139. Grace, Edwin J.: Tobacco smoking and cancer of the lung. *Am. J. Surg.*, 60: 361–364, 1943.
140. Graham, E. A., and J. J. Singer: Successful removal of an entire lung for carcinoma of bronchus. *J. A. M. A.*, 101: 1371–1374, 1933.
141. Graham, Evarts Ambrose: Died March 4, 1957, age 73 years. *J. A. M. A.*, 163: 1278, 1957.
142. Graham, Evarts A.: The problem of bronchiogenic carcinoma. *Surg. Clin. N. Am.*, 30: 1259–1277, 1950.
143. Greene, Harry S. N.: Hearings before a Subcommittee of the Committee on Government Operations. House of Representatives. Eighty-Fifth Congress, July 18–26, pp. 204–224. U. S. Government Printing Office, Washington, D.C., 1957.
144. Gregorius, Frederick: Lung cancer in the chromate industry. *Arch. Ind. Hyg. and Occup. Med.*, 5: 196–197, 1952.
145. Grosse, von Haus: One hundred years of lung cancer. *Arch. für Geschwulstforschung Band V*. Heft 4, 1953: 318–334.
146. Gsell, Otto: Tobacco and cancer. Clinical and statistical collections (with special consideration of Switzerland). *Oncol.*, 10: 157–186, 1957.
147. Gsell, O.: Klinische studien zur aetiologie des bronchialkarzinoms. *Dtsch. med. Wchnschr.*, 81: 496–501, 1956.
148. Guérin, M.: Tumeurs pulmonaires et cancer buccal chez le rat soumis a l'inhalation de fumée de cigarette. *Bull. L'Assn. fran. L'Etude du Cancer*, 46: 295–309, 1959.
149. Guiss, Lewis W.: Mass roentgenographic screening as a lung-cancer-control measure. *Cancer*, 8: 219–236, 1955.
150. Guiss, Lewis W.: A 5-year follow-up of roentgenographically detected lung cancer suspects. *Cancer*, 13: 82–90, 1960.
151. Guiss, Lewis W., and Patricia Kuenstler: A retrospective view of survey photofluorograms of persons with lung cancer. *Cancer*, 13: 91–95, 1960.
152. Guttman, Ruth J.: Two million volt irradiation therapy for inoperable carcinoma of the lung. *Cancer*, 8: 1254–1260, 1955.
153. Guyer, M. F., and P. E. Claus: Tumor of the lung in rats following injections of urethane (ethyl carbamate). *Cancer Res.*, 7: 342–345, 1947.
154. Haag, Harvey B., and Hiram R. Hanmer: Smoking habits and mortality among workers in cigarette factories. *Ind. Med. and Surg.*, 26: 559–562, 1957.
155. Haenszel, William, and Michael B. Shimkin: Smoking patterns and epidemiology of lung cancer in the United States: are they compatible? *J. Nat'l Cancer Inst.*, 16: 1417–1441, 1956.
156. Haenszel, William, Michael B. Shimkin, and Nathan Mantel: A retrospective study of lung cancer in women. *J. Nat'l Cancer Inst.*, 21: 825–842, 1958.
157. Haenszel, William, Michael B. Shimkin, and Herman P. Miller: Tobacco Smoking Patterns in the United States. Public Health Monograph No. 45. Public Health Service Publication No. 463. U. S. Government Printing Office, Washington, D. C., 1956.
158. Hamilton, John D., Ants Sepp, T. C. Brown, and F. W. Macdonald: Morphological changes in smokers' lungs. *Can. M. A. J.*, 77: 177–182, 1957.
159. Hamman, Louis: The diagnosis of carcinoma of the lung. *Am. Rev. Tbc.*, 28: 711–733, 1933.
160. Hammond, Arthur: Diagnosis and treatment of carcinoma of the lung with a statistical review of forty cases. *Arch. Otolaryn.*, 31: 780–807, 1940.
161. Hammond, E. Cuyler, and Daniel Horn: Smoking and death rates. Report of forty-four months of follow-up of 187,783 men. *J. A. M. A.*, 166: 1159–1172, 1294–1308, 1958.
162. Hammond, E. Cuyler, and David Horn: The relationship between human smoking habits and death rates. *J. A. M. A.*, 155: 1316–1328, 1954.
163. Hammond, E. Cuyler: Lung cancer death rates in England and Wales compared with those in the U. S. A. *Brit. Med. J.*, 2: 649–654, 1958.
164. Hammond, E. Cuyler, and Williard Machle: Environmental and Occupational Factors in the Development of Lung Cancer. Chap. 3 in *Pulmonary Carcinoma*. Pathogenesis, Diagnosis and Treatment. Ed., Edgar Mayer and Herbert C. Maier. New York University Press, New York, p. 45, 1956.
165. Harken, Dwight E., Harrison Black, Roy Clauss, and Robert E. Farrand: A single cervicomediastinal exploration for tissue diagnosis of intrathoracic disease. *N. Eng. J. Med.*, 251: 1041–1044, 1954.
166. Harris, William: Recent clinical experience with the grid in the x-ray treatment of advanced cancer. *Radiol.*, 58: 343–350, 1952.
167. Hartnett, T.: Tobacco industry scoffs at survey. *New York Times*, p. 44, col. 3, July 6, 1958.
168. Hayden, Charles: Early diagnosis of

bronchiogenic carcinoma. *Med. Times*, 76: 539–546, 1948.
169. Healey, John E., Jr., and John H. Gibbon, Jr.: Intrapericardial anatomy in relation to pneumonectomy for pulmonary carcinoma. *J. Thorac. Surg.*, 19: 864–874, 1950.
170. Herdan, G.: The increase in the mortality due to cancer of the lung in the light of the distribution of the disease among the different social classes and occupations. *Brit. J. Cancer*, 12: 492–506, 1958.
171. Heston, W. E., and Margaret K. Deringer: Induction of pulmonary tumors in guinea pigs by intravenous injection of methylcholanthrene and dibenzanthracene. *J. Nat'l Cancer Inst.*, 13: 705–718, 1952.
172. Heston, W. E.: Induction of pulmonary tumors in strain A mice with methylbis (β-chloroethyl) amine hydrochloride. *J. Nat'l Cancer Inst.*, 10: 125–130, 1949.
173. Heston, W. E.: Carcinogenic action of the mustards. *Cancer Res.*, 10: 224, 1950.
174. Higginson, John and A. G. Oettle: Cancer incidence in the Bantu and "Cape Colored" races of South Africa; report of a cancer survey in the Transvaal (1953–55). *J. Nat'l Cancer Inst.*, 24: 589–671, 1960.
175. Hill, A. Bradford, and E. Lewis Fanning: Studies in the incidence of cancer in a factory handling inorganic compounds of arsenic. I. Mortality experience in the factory. *Brit. J. Ind. Med.*, 5: 1–6, 1948.
176. Hodt, H. J., W. K. Sinclair, and D. W. Smithers: A gun for interstitial implantation of radioactive gold grains. *Brit. J. Radiol.*, 25: 419–421, 1952.
177. Hoffman, Frederick L.: Cancer and smoking habits. *Ann. Surg.*, 93: 50–67, 1931.
178. Hoffman, Erwin F., and Alexander G. Gilliam: Lung cancer mortality. Geographic distribution in the United States for 1948–1949. *Pub. Health Rep.*, 69: 1033–1042, 1954.
179. Holland, Robert H., Russell H. Wilson, Deloia Morris, Mary Sue McCall, and Henry Lanz: The effect of cigarette smoke on the respiratory system of the rabbit. *Cancer*, 11: 709–712, 1958.
180. Homann, E.: Lungenkrebs und lungensarkom. *Erg. inn. Med. Kinderh.*, 35: 206–254, 1929. Abst.; *Cancer Rev.*, 5: 540, 1930.
181. Horn, Helen A., and Harold L. Stewart: Spontaneous pulmonary tumor in the rat: report of a lesion. *J. Nat'l Cancer Inst.*, 12: 743–749, 1952.
182. Howitz, Melvin, and Charles W. Findlay: Supraclavicular lymph node biopsy in the diagnosis of intrathoracic lesions. *N. Y. St. J. Med.*, 57: 2065–2066, 1957.
183. Hruby, Allan J., and Henry C. Sweany: Primary carcinoma of the lung with special reference to incidence, early diagnosis and treatment. *Arch. Int. Med.*, 52: 497–540, 1933.
184. Hsieh, C. K., Shao-Hsun Wang, and Fa-Chu Chang: Primary carcinoma of the lung in the Chinese. Report of twenty-one cases. *Chinese M. J.*, 58: 381–407, 1940.
185. Hueper, W. C.: Environmental lung cancer. *Ind. Med. and Surg.*, 20: 49–62, 1951.
186. Hueper, W. C.: *Occupational Tumors and Allied Diseases*. Charles C Thomas, Springfield and Baltimore, 1942.
187. Hueper, W. C.: A quest into the environmental causes of lung cancer. Public Health Monograph #36. Public Health Service Publication #452, 1956.
188. Hueper, W. C.: Occupational and Environmental Pulmonary Cancer with Special Reference to Pneumoconiosis. Proc. Seventh Saranac Symposium on Pneumoconiosis, Saranac Lake, New York, Sept., 1952. *Am. Acad. Advancement Sci.*
189. Hueper, W. C.: Lung cancer and the tobacco smoking habit. *Ind. Med. and Surg.*, 23: 13–19, 1954.
190. Ibrahim, M.: Bronchogenic carcinoma in East Pakistan. *Dis. Chest*, 26: 286–294, 1954.
191. Ide, G., Valentina Suntzeff, and E. V. Cowdry: A comparison of the histopathology of tracheal and bronchial epithelium of smokers and non-smokers. *Cancer*, 12: 473–484, 1959.
192. Jacobs, Lewis G., James Yee, and Ivan A. May: Carcinoma of the lung. *Cancer*, 13: 362–369, 1960.
193. Jaffe, R. H.: The primary carcinoma of the lung. *J. Lab. and Clin. Med.*, 20: 1227–1237, 1935.
194. Jaffe, Werner G., and Rudolph Jaffe: Carcinogenic action of ethel urethane on rats. *Cancer Res.*, 7: 342–345, 1947.
195. Jeuther, A., H. Koeper, and H. Pionter: Die bösartigen geschwülste, lungenkrebse und tödlichen lungenembolien unter den Prager Leichenöffnungen 1894–1943. *Virch. Arch. f. Path. Anat.*, 314: 242–359, 1947.
196. Johnson, E. K., and Harry L. Reinhart: Necropsy incidence of cancer of the lung. *Ohio Med. J.*, 39: 1017–1018, 1943.
197. Kahlau, G.: Der lungenkrebs. *Ergebn. allg. Path. Anat.*, 37: 258–419, 1954. (Reference from Doll (85).)
198. Kawahata, Koretoki: Uber die gowerblich hervorger ufenen lungenkrebse bei Generator-Gas-Arbeitern in den Stahlwerken. *Gann*, 32: 367–370, 1938.
199. Kennaway, E. L., and R. E. Waller: Studies on cancer of the lung. *Acta Unio Internat. Contra Cancrum*, 9: 485–494, 1953.
200. Kennaway, E. L., and N. M. Kennaway: Studies of the incidence of cancer of the lung and larynx. *Brit. J. Cancer*, 5: 153–158, 1951.
201. Kennaway, E. L., and N. M. Kennaway: A further study of the incidence of cancer of the lung and larynx. *Brit. J. Cancer*, 1: 260–298, 1947.
202. Kennaway, N. M., and E. L. Kennaway: A study of the incidence of cancer of the lung and larynx. *J. Hyg.*, 36: 236–267, 1936.
203. Kennaway, Sir Ernest: Some questions on cancer of the lung, larynx, and urinary tract. *Brit. Med. J.*, 1: 299–306, 1957.
204. Kennaway, E. L.: The data relating to cancer in the publications of the General Register Office. *Brit. J. Cancer*, 4: 158–172, 1950.
205. Kirchoff, Helen, and R. H. Rigdon: Smoking habits of college students in Texas. *Tex. Rep. Biol. Med.*, 12: 292–299, 1954.
206. Kirchoff, Helen, and R. H. Rigdon: Smoking habits of 21,612 individuals in

Texas. *J. Nat'l Cancer Inst.*, 16: 1287–1304, 1956.

207. Kirchoff, Helen, and R. H. Rigdon: Cancer of the lung—the sex ratio. A review of the problem. *Tex. Rep. Biol. Med.*, 17: 29–48, 1959.
208. Kirklin, John W., John R. McDonald, O. Theron Clagett, Herman J. Moersch, and Robert P. Gage: Bronchogenic carcinoma: cell type and other factors relating to prognosis. *Surg. Gyn. Obst.*, 100: 429–438, 1955.
209. Klotz, Max O.: Primary carcinoma of the lung. *Am. J. Med. Sci.*, 196: 436–454, 1938.
210. Knudtson, Kenneth P.: The pathologic effects of smoking tobacco on the trachea and bronchial mucosa. *Am. J. Clin. Path.*, 33: 310–317, 1960.
211. Korteweg, R.: The age curve in lung cancer. *Brit. J. Cancer*, 5: 21–27, 1951.
212. Korteweg, R.: On cancer of the lung in the Netherlands. *Acta Unio Internat. Contra Cancrum*, 9: 589–597, 1953.
213. Kotin, Paul: The role of atmospheric pollution in the pathogenesis of pulmonary cancer: a review. *Cancer Res.*, 16, Supp. 4: 375–393, 1956.
214. Kotin, P., and H. L. Falk: Production of tumors in C57BL mice with atmosphere-extracted aliphatic hydrocarbons. *Proc. Am. Assn. Cancer Res.*, 2: 30, 1955. (Reference from Kotin (213).)
215. Kotin, Paul, Hans L. Falk, and Marilyn Thomas: Armatic hydrocarbons. III. Presence in the particulate phase of Diesel-engine exhausts and the carcinogenicity of exhaust extracts. *Arch. Ind. Health*, 11: 113–120, 1955.
216. Koulumies, Marja: Smoking and pulmonary carcinoma. *Acta Radiol.* 39: 256–260, 1953.
217. Kretz, J.: Cancer of the lung in Vienna. *Acta Unio Internat. Contra Cancrum*, 9: 542–551, 1953.
218. Kreyberg, Leiv: Lung tumours: histology, aetiology and geographic pathology. *Acta Union Internat. Contre le Cancer*, 15: 78–95, 1959.
219. Kreyberg, Leiv: Occurrence and aetiology of lung cancer in Norway in the light of pathological anatomy. *Brit. J. Prev. and Soc. Med.*, 10: 145–158, 1956.
220. Kreyberg, L.: The geographical distribution of histological subgroups of primary epithelial lung tumours in Norway. *Brit. J. Cancer*, 8 :599–604, 1954.
221. Kreyberg, L.: One hundred consecutive primary epithelial lung tumours. *Brit. J. Cancer*, 6: 112–119, 1952.
222. Kuroda, Shizuka: Occupational pulmonary cancer of generator gas workers. *Ind. Med.*, 6: 304–306, 1937.
223. Kuschner, M., S. Laskin, E. Cristofano, and N. Nelson: Experimental carcinoma of the Lung. Proc. 3rd Nat'l Cancer Conference, Detroit, Mich., June, 1956, pp. 485–495. J. B. Lippincott Company, Philadelphia and Montreal.
224. Lambret, Oscar, H. Malatray, and J. Driessens: La Chirugie du Cancer du Poumon. Masson et Cie, editeurs. *Libraires de L'academie de Medecine*, Paris, 1936.
225. Larson, P. S., H. B. Haag, and H. Silvette: Measurement of tobacco smoking. *Med. Times*, 88: 417–429, 1960.
226. Lecoeur, Jacques: Les Maladies des Bronches. Etude Anatomique Physiopathologique, Clinique et Therapeutique. Vigot Freres, Editeurs, Paris, 1950.
227. Leddy, Eugene T., and Herman J. Moersch: Roentgen therapy for bronchiogenic carcinoma. *J. A. M. A.*, 115: 2239–2242, 1940.
228. Lehmann, K. B.: 1st Grund zu einer besonderen Beunruhigung des Auftretens von Lungenkrebs bei Chromatarbeitern vorhanden? Zbl. Gewhyg., 9: 168–170, 1932.
229. Leiter, Joseph, Michael B. Shimkin, and M. J. Shear: Production of subcutaneous sarcomas in mice with tars extracted from atmospheric dusts. *J. Nat'l Cancer Inst.*, 3: 155–165, 1942.
230. Leiter, Joseph, and M. J. Shear: Production of tumors in mice with tars from city air dusts. *J. Nat'l Cancer Inst.*, 3: 167–174, 1942.
231. Leuchtenberger, Cecilie, Rudolph Leuchtenberger, Paul F. Doolin, and Patricia Shaffer: A correlated histological, cytological, and cytochemical study of the tracheobronchial tree and lungs of mice exposed to cigarette smoke. I. Bronchitis with atypical epithelial changes in mice exposed to cigarette smoke. *Cancer*, 11: 490–506, 1958.
232. Leuchtenberger, Cecilie, Rudolph Leuchtenberger, William Zebrun, and Patricia Shaffer: A correlated histological, cytological, and cytochemical study of the tracheobronchial tree and lungs of mice exposed to cigarette smoke. II. Varying responses of major bronchi to cigarette smoke, absence of bronchogenic carcinoma after prolonged exposure, and disappearance of bronchial lesions after cessation of exposure. *Cancer*, 13: 721–732, 1960.
233. Levin, Morton L., Hyman Goldstein, and Paul R. Gerhardt: Cancer and tobacco smoking. A preliminary report. *J. A. M. A.*, 143: 336–338, 1950.
234. Levin, Morton L.: Etiology of lung cancer; present status. *N. Y. St. J. Med.*, 54: 769–777, 1954.
235. Levin, Morton L., William Haenszel, Benjamin Carroll, Paul R. Gerhardt, Vincent H. Handy, and Samuel C. Ingraham: Cancer incidence in urban and rural areas of New York State. *J. Nat'l Cancer Inst.*, 24: 1243–1257, 1960.
236. Lezza, Felice: Carcinoma Bronchiale. Prefazione del Prof. P. Valdoni. *Sansoni Edizioni Scientifiche, Firenze*, 1950.
237. Liberti, R., and G. Stella: Il Cancro Primitivo del Polmone. *Editrice Archivio di Tisiologia, Napoli*, 1949.
238. Lickint, Fritz: Der bronchialkrebs der raucher. *Munch. Med. Wchnschr.*, 82: 1232–1234, 1935.
239. Linckint, F.: Aetiologie und Prophylaxe des Lungenkrebses. *Steinkopff, Dresden*, 1953.
240. Lickint, F.: Tabak und tabaksrauch als ätiologischen faktor des carcinoma. *Ztschr. f. Krebs.*, 30: 349–365, 1929–1930.
241. Little, Clarence Cook: Hearings before a Subcommittee of the Committee on Government Operations. House of Representatives Eighty-Fifth Congress, July 18–26, pp. 34–61, U. S. Government Printing Office, Washington, 1957.
242. Lindskog, Gustaf E., and William E. Bloomer: Bronchogenic carcinoma. A comparison of 2 consecutive series of one hundred cases each. *Cancer*, 1: 234–237, 1948.

243. Livingood, L. E.: Tumors in the mouse. *Johns Hopkins Hosp. Bull.*, 7: 177–178, 1896.
244. Loizaga, Niceto S.: Del Carcinoma Primivito Broncopulmonar. *Liberia y Editorial "El Ateneo,"* 1938.
245. Loken, A. C.: Lungencarcinom hos nikkelarbeidere. *Tidsskr. norske Laegeforen.*, 70: 376, 1950. (Reference from Doll (85).)
246. Lorenz, Egon, Harold L. Stewart, John H. Daniel, and Clifford V. Nelson: The effects of breathing tobacco smoke on strain A mice. *Cancer Res.*, 3: 123, 1943.
247. Lorenz, Egon, Michael B. Shimkin, and Harold L. Stewart: Preparations of dispersions of carcinogenic hydrocarbons and hormones with the aid of dioctyl ester of sodium sulfosuccinate (Aerosol O.T.). *J. Nat'l Cancer Inst.*, 1: 355–360, 1940.
248. Lorenz, Egon, Walter E. Heston, Margaret K. Deringer, and Allen B. Eschenbrenner: Increase in incidence of lung tumors in strain A mice following long-continued irradiation with gamma rays. *J. Nat'l Cancer Inst.*, 6: 348–353, 1946.
249. Lowe, C. R.: An association between smoking and respiratory tuberculosis. *Brit. Med. J.*, 2: 1081–1086, 1956.
250. Lynch, Kenneth M., and W. Atmar Smith: Pulmonary asbestosis. III. Carcinoma in lung in asbesto-silicosis. *Am. J. Cancer*, 24: 56–64, 1935.
251. Macdonald, Ian G.: Hearings before a Subcommittee of the Committee on Government Operations. House of Representatives. Eighty-Fifth Congress, July 18–26, pp. 224–240. U. S. Government Printing Office, Washington, D. C., 1957.
252. Machle, Willard, and Frederick Gregorius: Cancer of the respiratory system in the United States chromate-producing industry. *Pub. Health Rep.*, 63: 1114–1127, 1948.
253. Macklin, Madge Thurlow: Has a real increase in lung cancer been proved? *Ann. Int. Med.*, 17: 308–324, 1942.
254. Macklin, Madge Thurlow: Pitfalls in dealing with cancer statistics, especially as related to cancer of the lung. *Dis. Chest*, 14: 525–533, 1948.
255. Macklin, Madge Thurlow, and Charles C. Macklin: Does chronic irritation cause primary carcinoma of the human lung? *Arch. Path.*, 30: 924–955, 1940.
256. Mandell, Edward: Bronchiogenic carcinoma of the lung. *J. Lancet*, 67: 157–166, 1947.
257. Marks, Hirsch: A new approach to the roentgen therapy of cancer with the use of a grid. *J. Mt. Sinai Hosp.*, 17: 46–48, 1950.
258. Marks, Hirsch: Clinical experience with irradiation through a grid. *Radiol.*, 58: 338–341, 1952.
259. Mason, George A.: Cancer of the lung. Review of a thousand cases *Lancet*, 2: 587–591, 1949.
260. Matz, Philip B.: The incidence of primary bronchiogenic carcinoma. *J. A. M. A.*, 111: 2086–2092, 1938.
261. McConnell, R. B., K. C. T. Gordon, and Thelwall Jones: Occupational and personal factors in the aetiology of carcinoma of the lung. *Lancet*, 2: 651–656, 1952.
262. McDonald, John R., Robert P. McBurney, John C. Carlisle, and Matthew M. Patton: The significance of cell types in bronchogenic carcinoma. *J. Thorac. Surg.*, 22: 62–93, 1951.
263. McDonald, Stuart, Jr., and D. L. Woodhouse: On the nature of mouse lung adenomata, with special reference to the effects of atmospheric dust on incidence of these tumours. *J. Path. and Bact.*, 54: 1–12, 1942.
264. McEwen, D. S.: Bronchogenic carcinoma. *Manitoba Med. Rev.*, 28: 235–237, 1948.
265. McKenzie, Alan: Diagnosis of cancer of lung and stomach. *Brit. Med. J.*, 2: 204–207, 1956.
266. McNally, Wm. D.: The tar in cigarette smoke and its possible effects. *Am. J. Cancer*, 16: 1502–1514, 1932.
267. McPhee, J. Gordon, and Wm. R. LaCroix: A statistical analysis of 1,214 cases of carcinoma. *Can. M. A. J.*, 54: 573–584, 1946.
268. Medical Annotations. The tobacco controversy. *Lancet*, 1: 770, 1872.
269. Medical Notes in Parliament. Smoking and lung cancer. *Brit. Med. J.*, 1: 465, 1954.
270. Medical Research Council. Tobacco smoking and cancer of the lung. *Brit. Med. J.*, 1: 1523–1524, 1957.
271. Menne, Frank R., and Melvin W. Anderson: Bronchiogenic carcinoma. Incidence in the Pacific Northwest with a commentary on eighty-four cases. *J.A.M.A.*, 117: 2215–2222, 1941.
272. Merewether, E. R. A.: Annual Report of the Chief Inspector of Factories for the Year 1947. *H. M. S. O.*, London. (Reference from Doll (85).)
273. Metropolitan Life Insurance Co. Cancer of the lung a growing health problem. *Stat. Bull.*, 20: (11) 7–9, 1939.
274. Mills, Clarence A., and Marjorie Mills Porter. Tobacco smoking habits and cancer of the mouth and respiratory system. *Cancer Res.*, 10: 539–542, 1950.
275. Mills, Clarence A., and Marjorie M. Porter: Tobacco-smoking habits in an American city. *J. Nat'l Cancer Inst.*, 13: 1283–1297, 1953.
276. Milmore, Benno K.: Trends of lung-cancer mortality in the United States: some limitations of available statistics. *J. Nat'l Cancer Inst.*, 16: 267–284, 1955.
277. Moersch, Herman J., and John R. McDonald: The significance of cell types of bronchogenic carcinoma. *Dis. Chest*, 23: 621–633, 1953.
278. Monlux, William S.: Primary neoplasms in domestic animals. *Southwestern Vet.*, 6: 131–133, 1952.
279. Morrison, S. L.: Occupational mortality in Scotland. *Brit. J. Ind. Med.*, 14: 130–132, 1957.
280. Mostofi, F. K., and C. D. Larsen: Pulmonary lesions induced in Wistar rats by urethane. *Am. J. Path.*, 25: 807–838, 1949.
281. Muller, Franz Hermann: Tobacco misuse and lung carcinoma. *Ztschr. f. Krebs.*, 59: 57–84, 1939.
282. Muller, J. H., and P. H. Rossier: De l'emploi d'istopes radioactifs artificiels, dans le but d'exercer un effet radiobiologique localise. *Experientia*, 3: 75–76, 1947.
283. Muller, J. H., and P. H. Rossier: A new method for the treatment of cancer of the lungs by means of artificial radioactivity. *Acta Radiol.*, 35: 449–468, 1951.
284. Murphy, James B., and Ernest Sturm:

Primary lung tumors in mice following the cutaneous application of coal tar. *J. Exper. Med.*, 42: 693–700, 1925.
285. Nagayo, Mataro: Statistical studies on cancer in Japan. *Gann*, 27: 1–176, 1933.
286. Nasr, A. Loutfy Aboul: Aetiological factors in cancer of the lung. *J. Egyptian M. A.*, 40: 55–62, 1957.
287. Nau, Carl A., Jack Neal, and Vernie Stembridge: A study of the physiological effects of carbon black. *Arch. Ind. Health*, 17: 21–28, 1958.
288. Neely, J. Marshall: Primary carcinoma of the lung. *Nbr. St. Med. J.*, 20: 247–252, 1935.
289. Netherlands Ministry of Social Affairs and Public Health (1957): Report Concerning the Connexion between Smoking and Lung Cancer. *The Hague*: Press Notice No. 1233, Aug. 3, 1957. (Reference from Doll (95).)
290. Nettleship, Anderson, Paul S. Henshaw, and Henry L. Meyer: Induction of pulmonary tumors in mice with ethyl carbonate (urethane). *J. Nat'l Cancer Inst.*, 4: 309–319, 1943.
291. Neurdenburg, M. G.: Some statistical information on cancer of the lung in the Netherlands. *Acta Unio Internat. Contra Cancrum*, 9: 580–588, 1953.
292. Neyman, Jerzy: Statistics—servant of all sciences. *Sci.*, 122: 401–406, 1955.
293. Nickson, James J., and Bernard Roswitt: Radiotherapy in Lung Cancer. Chap. 13 in *Pulmonary Carcinoma*. Ed., Edgar Mayer and Herbert C. Maier. New York University Press, New York, 1956.
294. Niskanen, K. O.: Observations on metaplasia of the bronchial epithelium and its relation to carcinoma of the lung. Patho-anatomical and experimental researches. *Acta. Path. et. Micro. Scan.*, Supp. 80: 1–80, 1949.
295. Nohl, H. C.: An investigation into the lymphatic and vascular spread of carcinoma of the bronchus. *Thorax*, 11: 172–185, 1956.
296. Nordmann, Martin, and Adolf Sorge: Lungenkrebs durch Asbeststaub im Tierversuch. *Ztschr. f. Krebs.*, 51: 168–182, 1941.
297. Ochsner, Alton and Michael DeBakey: Carcinoma of the lung. *Arch. Surg.*, 42: 209–258, 1941.
298. Ochsner, Alton, and Michael DeBakey: Primary pulmonary malignancy. Treatment by total pneumonectomy. Analysis of 79 collected cases and presentation of 7 personal cases. *Surg. Gyn. Obst.*, 68: 435–451, 1939.
299. Ochsner, A.: Tremendous increase in cancer of the lung. *Life and Health*, 34: 6–7, 1946.
300. Ochsner, Alton, Michael DeBakey, and J. Leonard Dixon: Primary cancer of the lung. *J. A. M. A.*, 135: 321–327, 1947.
301. Ochsner, Alton, C. Jack Ray, and Page W. Acree: Cancer of the lung. A review of experiences with 1,457 cases of bronchogenic carcinoma. *Am. Rev. Tbc.*, 70: 763–783, 1954.
302. Ochsner, Alton, and Michael DeBakey: Surgical considerations of primary carcinoma of the lung. Review of the literature and report of 19 cases. *Surg.*, 8: 992–1023, 1940.
303. Ochsner, Alton, Michael DeBakey, Charles E. Dunlap, and Irving Richman: Primary pulmonary malignancy. Factors influencing survival. *J. Thorac. Surg.*, 17: 573–599, 1948.
304. Onuigbo, W. I. B.: Lung cancer in the nineteenth century. *Med. History*, 3: 69–77, 1959.
305. Ormerod, F. C.: The pathology and treatment of carcinoma of the bronchus. *J. Laryn. and Otol.*, 52: 733–745, 1937.
306. Ormerod, F. C.: Some notes on the treatment of carcinoma of the bronchus. *J. Laryn. and Otol.*, 56: 1–10, 1941.
307. Ormerod, F. C.: The late results of treatment, by interstitial irradiation, of one hundred cases of carcinoma of the bronchus. *J. Laryn. and Otol.*, 67: 406–416, 1953.
308. Ornstein, George G., and Laurence Lercher: Primary pulmonary carcinoma. *Dis. Chest*, 27: 414–420, 1955.
309. Osler, William: *The Principles and Practice of Medicine*. p. 556. D. Appleton & Co., New York, 1892.
310. Overholt, Richard H., and Lazard Langer: The Technique of Pulmonary Resection. Chas. C Thomas, Springfield, Ill. 1949.
311. Overholt, Richard H., and Ivan C. Schmidt: Survival in primary carcinoma of the lung. *New Eng. J. Med.*, 240: 491–497, 1949.
312. Overholt, Richard H.: Curability of primary carcinoma of lung. Early recognition and management. *Surg. Gyn. Obst.*, 70: 479–490, 1940.
313. Pascua, M.: Increased mortality from cancer of the respiratory system. *Bull. World Hlth. Org.*, 12: 687–704, 1955.
314. Passey, R. D., and J. McD. Holmes: The incidence of intrathoracic neoplasia in the teaching hospitals of Great Britain 1894–1928. *Quart. J. Med.*, N. S. 4: 321–344, 1935.
315. Passey, R. D.: Cigarette smoking as a primary cause of lung cancer? *Med. Sci.*, 2: #9, 6–8, 1957.
316. Passey, R. D., F. Bergel, G. E. Lewis, E. M. F. Roe, F. C. Middleton, E. Boyland, B. M. G. Pratt, I. Sim, and I. Hieger: Cigarette Smoking and Cancer of the Lung. Thirty-third Annual Report of the British Empire Cancer Campaign Covering the Year 1955, pp. 59–61.
317. Passey, R. D.: Individual Reports. Thirty-fourth Annual Report of the British Empire Cancer Campaign Coverin the Year 1956, pp. 449–451.
318. Passey, R. D.: Report of the Department of Experimental Pathology and Cancer Research. University of Leeds for the year ending 31st December, 1952. Yorkshire Council of the British Empire Cancer Campaign Reports and Accounts, 1952–1953.
319. Passler, H.: Ueber das primare carcinom des lunge. *Virch. Arch. f. Path. Anat.*, 145: 191–278, 1896.
320. Peacock, P. R.: Experimental cigarette smoking by domestic fowls. *Brit. J. Cancer*, 9: 461–463, 1955.
321. Peery, Thomas M.: Evaluation of the apparently increased incidence of primary carcinoma of the lung. *Arch. Path.*, 29: 625–632, 1940.
322. Pekelis, E.: Contributo allo studie anatomo-pathologico dei carcinomi primitivi del pulmone. Tumori, 17: 33–81, 1931. Abst., *Cancer Rev.*, 7: 43, 1932.
323. Peller, Sigismund: Lung cancer among

mine workers in Joachimsthal. *Human Biol.*, 11: 130–143, 1939.
324. Pfeil, E.: Lungentumoren als Berufserkrankung in Chromatbetrieben. *Dtsch. Med. Wschr.*, 61: 1197–1200, 1935.
325. Plainos, Theodoros K.: Primary Lung Cancer. *Adelphon Euth. Kobane*, Athens, 1948.
326. Pohlen, Kurt, and Haven Emerson: Errors in clinical statements of causes of death. *Am. J. Pub. Health*, 32: 251–260, 1942.
327. Porta, Guiseppe Della, Leonard Kolb, and Philippe Shubik: Induction of tracheobronchial carcinomas in the Syrian golden hamsters. *Cancer Res.*, 18: 592–597, 1958.
328. Portmann, U. V.: The role of roentgen therapy in the treatment of bronchiogenic carcinoma. *Cleveland Clin. Quart.*, 7: 119–122, 1940.
329. Price Thomas, Sir Clement: Conservative resection of the bronchial tree. *J. Roy. Coll. Surg. Edinburgh*, 1: 169–186, 1956.
330. Proetz, Arthur: Some preliminary experiments in the study of cigarette smoke and its effects upon the respiratory tract. *Ann. Otol. Rhinol. and Laryngol.*, 48: 176–194, 1939.
331. Raffle, P. A. B.: The health of the worker. *Brit. J. Ind. Med.*, 14: 73–80, 1957.
332. Rakov, A. I.: Some aspects of the problem of lung cancer. *Prob. Oncol.*, 3: 403–409, 1957.
333. Randig, K.: The etiology of pulmonary cancer. Questions and results. *Dtsch. Med. Wschr.*, 80: 718–724, 1955.
334. Registrar General for Scotland (1956): Annual Report 1955, No. 101, Appendix IX: Occupational Mortality, pp. 75–133. *Edinburgh: H. M. S. O.*
335. Registrar-General's Statistical Review of England and Wales for the Year 1937, p. 182. *H. M. S. O.*, London, 1940.
336. Registrar-General's Statistical Review of England and Wales for the Year 1956. Part III. Commentary. *H. M. S. O.*, London, 1958.
337. Reinhoff, William Francis, Jr.: A clinical analysis and follow-up study of five hundred and two cases of carcinoma of the lung. *Dis. Chest*, 17: 33–54, 1950.
338. Rhoads, Cornelius P.: Nitrogen mustards in the treatment of neoplastic disease. *J. A. M. A.*, 131: 656–658, 1946.
339. Rigdon, R. H.: Cancer of the lung before 1900. A historical review. *Tex. Rpt. Biol. Med.*, 13: 993–1099, 1955.
340. Rigdon, R. H., and Helen Kirchoff: Cancer of the lung from 1900 to 1930. *Surg. Gyn. Obst.*, 107: 105–118, 1958.
341. Rigdon, R. H., and Helen Kirchoff: A consideration of some of the theories relative to the etiology and incidence of lung cancer. *Tex. Rep. Biol. Med.*, 10: 76–91, 1952.
342. Rigdon, R. H., and Helen Kirchoff: Smoking and disease. A study based upon 12,050 individuals. *Tex. Rep. Biol. Med.*, 16: 116–132, 1958.
343. Rigdon, R. H.: Hearings before a Subcommittee of the Committee on Government Operations. House of Representatives. Eighty-Fifth Congress, July 18–26, 1957, pp. 114–131. U. S. Government Printing Office, Washington, D. C., 1957.
344. Rigdon, R. H.: Consideration of the relationship of smoking to lung cancer: with a review of the literature. *Southern Med. J.*, 50: 524–532, 1957.
345. Rigdon, R. H., and Helen, Kirchoff: Accuracy of death certificates for establishing the frequency of cancer as shown by autopsy. *Tex. Rep. Biol. Med.*, 9: 652–659, 1951.
346. Rigdon, R. H.: The smoking controversy, *J. A. M. A.*, 173: 293–294, 1960.
347. Rigdon, R. H., and Paul Brindley: Primary cancer of the lung in Texas. *Tex. St. J. Med.*, 46: 885–889, 1950.
348. Rigdon, R. H., Helen Kirchoff, and Norma Martin: Where cancer of the lung occurs in Texas. *Tex. Rep. Biol. Med.*, 13: 162–169, 1955.
349. Rigdon, R. H., and Helen Kirchoff: Frequency of cancer of the lung in all malignancies studied at autopsy. *Southern Med. J.*, 44: 506–512, 1951.
350. Rigdon, R. H.: Effect of tobacco condensate on respiratory tract of white Pekin ducks. *Arch. Path.*, 69: 55–63, 1960.
351. Rigdon, R. H.: Effect of methylcholanthrene on the respiratory tract of the white Pekin duck. *Arch. Path.*, 68: 578–588, 1959.
352. Rigdon, R. H., and Helen Kirchoff: Smoking and cancer of the lung. Let's review the facts. *Tex. Rep. Biol. Med.*, 11: 715–727, 1953.
353. Ringertz, N.: Environmental factors and smoking in the causation of cancer of the lung. *Schweiz. Zeit. f. Path. und Bakt.*, 18: 866–884, 1955.
354. Rockey, Edward Ernest, Marvin Kuschner, Alvin I. Kosak, and Edgar Mayer: The effect of tobacco tar on the bronchial mucosa of dogs. *Cancer*, 11: 466–472, 1958.
355. Roffo, A. H.: Tobacco as a cancerigen. *Bol. del Inst. de Med. Exp. para el estud. y trat. del Cancer*, 13: 287–336, 1936.
356. Rosahn, Paul D.: Incidence of primary carcinoma of the lung. A review of Yale autopsy protocols, 1917 to 1937. *Arch Path.*, 29: 649–664, 1940.
357. Rosahn, Paul D.: The incidence of primary carcinoma of the lung. *Am. J. Med. Sci.*, 179: 803–811, 1930.
358. Rosemond, George P., Emory W. Burnett, and John H. Hall: Diagnosis of carcinoma of the lung. *J. Internat. Coll. Surg.*, 14: 100–106, 1950.
359. Rosenblatt, Milton B., and James R. Lisa: Cancer of the Lung. *Pathology, Diagnosis and Treatment*. Oxford University Press, New York, 1956.
360. Rosenblatt, Milton B.: Hearings before a Subcommittee of the Committee on Government Operations. House of Representatives. Eighty-Fifth Congress, July 18–26, 1957. Letter to Surgeon General, pp. 753–754. U. S. Government Printing Office, Washington, D. C., 1957.
361. Rothgerber, Jean-Antoine: Contribution a L'étude Démographique-Etiologique et Pathogénique du Cancer Primitif du Poumon. These. Univ. de Nancy. *Imprimerie J. Grandidier*, Schilitgheim, 1937.
362. Rouvière, H.: Anatomie Des Lymphatiques del l'Homme. *Masson et C. Editeurs*. Paris, 1932.
363. Russ, Sidney: Smoking and Its Effects. *Hutchinson's Scientific and Technical Publications*. The Anchor Press, Ltd., Tiptree Essex, 1955.

364. Sackrin, Seymour M., and Arthur G. Conover: Tobacco Smoking in the United States in Relation to Income. Marketing Research Report No. 189. U. S. Dept. of Agriculture. Agricultural Marketing Service. Washington, D. C., July, 1957.
365. Sadowsky, Doris A., Alexander G. Gilliam, and Jerome Cornfield: The statistical association between smoking and carcinoma of the lung. *J. Nat'l Cancer Inst.*, 13: 1237–1258, 1953.
366. Salzer, G., M. Wenzl, R. H. Jenny, and A. Stangl: Das Bronchuscarcinom. *Springer-Verlag*, Vienna, 1952.
367. Sanderud, K.: Squamous metaplasia of the respiratory tract epithelium: an autopsy study of 214 cases. I. Incidence, age and sex distribution. *Acta Path. Micro. Scan.*, 42: 247–267, 1958. II. Relation to tobacco smoking, occupation and residence. *Ibid.*, 43: 47–61, 1958. III. Relation to disease. *Ibid.*, 44: 21–31, 1958. IV. Relation to bronchial carcinoma. *Ibid.*, 44: 329–344, 1958.
368. Saxton, John A., Jr., Gladys A. Sperling, LeRoy A. Barnes, and Clive M. McCay: The influence of nutrition upon the incidence of spontaneous tumors of the Albino rat. *Acta Unio Internat. Contra Cancrum*, 6: 423–431, 1948–1950.
369. Schabad, L. M.: Studien über primäre Lungengeschwüste bei Mausen und ihr Verhalten zum Steikohlenteer, als cancerogenem Faktor. *Ztschr. f. Krebs.*, 30: 24–59, 1929.
370. Schairer, E., and E. Schoeniger: Cancer of the lungs and tobacco consumption. *Ztschr. f. Krebs.*, 54: 261–269, 1943.
371. Schinz, H. R., and H. Steinmann: Uterusadenome und lungencarcinom bei einem mit methylcholanthren behandelten kaninchen. *Oncol.*, 2: 185–188, 1949.
372. Schrek, Robert, Lyle A. Baker, George P. Ballard, and Sidney Dolgoff: Tobacco smoking as an etiologic factor in disease. I. Cancer. *Cancer Res.*, 10: 49–57, 1950.
373. Schwartz, Daniel, and Pierre F. Denoix: L'Enquete Francaise sur L'Etiologie du Cancer Broncho-pulmonaire. *Role du Tabac. Sem. Hop.* Paris, 33: 424–437, 1957.
374. Schwippert, Harry, and Joseph E. MacManus: A histologic study of routine scalene node biopsies. *Surg.*, 42: 533–535, 1957.
375. Science in the News. Jury decides cigarettes caused lung cancer but company is not liable. *Sci.*, 132: 404, 1960.
376. Seheult, R.: Observations on the incidence of cancer in Trinidad. *Caribbean M. J.*, 7: 72–79, 1945.
377. Selbie, F. R., and A. C. Thackray: Lung adenomas induced by urethane in CBA mice. *Brit. J. Cancer*, 2: 380–385, 1948.
378. Sellors, T. Holmes: Results of surgical treatment of carcinoma of the lung. *Brit. Med. J.*, 1: 445–448, 1955.
379. Shekhter, I. A.: Difficulties and errors in the x-ray diagnosis of lung cancer. *Prob. Oncol.*, 3: 590–596, 1957.
380. Shik, R. G.: Bronchogenic carcinoma. *Am. Rev. Soviet Med.*, 4: 142–147, 1946.
381. Shimkin, Michael B.: *Pulmonary Tumors in Experimental Animals, in Advances in Cancer Research*. Ed., Jesse P. Greenstein and Alexander Haddow, Vol. III, pp. 223–267. Academic Press, New York, 1955.
382. Shimkin, Michael B.: Induced pulmonary tumors in mice. I. Susceptibility of seven strains of mice to the action of intravenously injected methylcholanthrene. *Arch. Path.*, 29: 229–238, 1940.
383. Sikl, H.: The present status of knowledge about the Jachymov disease. (Cancer of the lungs in the miners of the radium mines.) *Acta Unio Internat. Contra Cancrum*, 6: 1366–1375, 1948.
384. Simons, Edwin J.: *Primary Carcinoma of the Lung*. The Yearbook Publishers, Inc., Chicago, 1937.
385. Sison, A. G., and C. Monserrat: Primary malignant tumors of the lung, their incidence in The Philippines, and their most common clinical manifestation. *J. Philippine Island M. A.*, 7: 422–438, 1927.
386. Sitbon, J., and A. Hadida: Role du tabac dans l'evolution des cancers larynges et broncho-pulmonaires. *Bull. Alger. Carcinol.*, 6: 136–147, 1953.
387. Sjolte, I. P.: Primäre maligne tumoren der lungen bei tieren. Carcinoma et carcino-sarcoma pulmonum primum. *Arch. f. Path. anat. Physiol.*, 312: 35–63, 1944.
388. Slye, Maud, Harriet. F. Holmes, and H. Gideon Wells: The primary spontaneous tumors of the lungs in mice. Studies on the incidence and inheritability of spontaneous tumors in mice. *J. Med. Res.*, 30: 417–442, 1914.
389. Smart, Joseph, and Gwen Hilton: Radiotherapy of cancer of the lung. Results in a selected group of cases. *Lancet*, 1: 880–881, 1956.
390. Smith, R. Abbey: The results of raising the resectability rate in operations for lung carcinoma. *Thorax*, 12: 79–86, 1957.
391. Smith, William E.: Survey of some current British and European studies of occupational tumor problems. *Arch. Ind. Hyg. and Occup. Med.*, 5: 242–263, 1952.
392. Smith, William E.: An Evaluation of Claims for Occupational Factors in Cancer of the Lungs. Symposium on the Endemiology of Cancer of the Lung, Louvain, Belgium. *Acta Unio Internat. Contra Cancrum*, 9: 476–484, 1953.
393. Smith, William E.: Lung cancer with special reference to experimental aspects. *Arch. Ind. Hyg. and Occup. Med.*, 5: 209–217, 1952.
394. Smith, William E.: The neoplastic potentialities of mouse embryo tissues. V. The tumors elicited with methylcholanthrene from pulmonary epithelium. *J. Exper. Med.*, 91: 87–104, 1950.
395. Smith, William E.: The tissue transplant technic as a means of testing materials for carcinogenic action. *Cancer Res.*, 9: 712–723, 1949.
396. Smith, William E., and Peyton Rous: The neoplastic potentialities of mouse embryo tissues. IV. Lung adenomas in baby mice as result of prenatal exposure to urethane. *J. Exper. Med.*, 88: 529–554, 1948.
397. Smithers, D. W.: Facts and fancies about cancer of the lungs. *Brit. Med. J.*, 2: 1235–1247, 1953.
398. Smithers, D. W.: Radiotherapy. Chap. 22 in Carcinoma of the Lung. Ed., J. R. Bignall. Vol. I. *Monographs on Neoplastic Diseases*, gen. ed., D. W. Smithers. E. & S. Livingstone, Ltd., Edinburgh and London, 1958.
399. Smoking and Health. Joint report of the

study group on smoking and health. *Sci.*, 125: 1129–1133, 1957.
400. Snegireff, Leonid S., and Olive M. Lombard: Arsenic and cancer. Observations in the metallurgical industry. *Arch. Ind. Hyg. and Occup. Med.*, 4: 199–205, 1951.
401. State Medical Research Council of Sweden, 1958. (Reference from World Health Organization Technical Report Series No. 192 (448).)
402. Steiner, Paul E.: Incidence of primary carcinoma of the lung with special reference to its increase. *Arch. Path.*, 37: 185–195, 1944.
403. Steiner, Paul E., E. M. Butt, and Hugh A. Edmondson: Pulmonary carcinoma revealed at necropsy, with reference to increasing incidence in the Los Angeles County Hospital. *J. Nat'l Cancer Inst.*, 11: 497–510, 1950.
404. Steiner, Paul E.: Etiological problem in human lung cancer. *Cancer*, 9: 664–666, 1956.
405. Steiner, Paul E.: The conditional biological activity of the carcinogens in carbon blacks and its elimination. *Cancer Res.*, 14: 103–110, 1954.
406. Stewart, Harold L.: Pulmonary Tumors in Mice. Chap. 2 in *The Physiopathology of Cancer*, Ed. Freddy Homburger, 2nd ed., Paul B. Hoeber, Inc., New York, N. Y., 1953.
407. Sticker, Anton: Ueber den Krebs der Thiere insbesondere über die Empfanglichkeit der verschiedenen Hausthierarten und die Unterschiede des Thier-und Menschenkrebses. *Arch. f. Klin. Chir.*, 65: 616–696, 1902.
408. Stocks, P.: Endemiology of cancer of the lung in England and Wales. *Brit. J. Cancer*, 6: 99–111, 1952.
409. Stocks, Percy: Statistics of cancer of the lung. *J. Fac. Radiol.*, 6: 166–173, 1955.
410. Stocks Percy, and John M. Campbell: Lung cancer death rates among non-smokers and pipe and cigarette smokers. *Brit. Med. J.*, 2: 923–929, 1955.
411. Storer. Carcinoma of right lung with symptoms resembling those of hydrothorax. *Am. J. Med. Sci.*, 21: 46–47, 1851.
412. Strachan, A. Sutherland: Observations on the incidence of malignant disease in South African Natives. *J. Path. and Bact.*, 39: 209–211, 1934.
413. Studies in Methods. Handbook of Vital Statistics Methods. Series F. #7. Statistical Office of United Nations, N. Y., 1955.
414. Sweany, Henry C.: Primary bronchiogenic carcinoma; incidence, pathogenesis and diagnosis. *Ann. Otol. Rhinol. Laryngol.*, 43: 561–571, 1934.
415. Symposium on the Endemiology of Cancer of the Lung. University of Louvain, Belgium. *Acta Unio Internat. Contra Cancrum*, 9: 437–636, 1953.
416. Talbott, John H.: Smoking and lung cancer. *J. A. M. A.*, 171: 2104, 1959.
417. Taylor, A. Brian, and J. A. H. Waterhouse: Prognosis for bronchial carcinoma. *Thorax*, 5: 257–267, 1950.
418. Temple, Lewis A., D. H. Willard, S. Marks, and W. J. Bair: Induction of lung tumours by radioactive particles. *Nature*, 183: 408–409, 1959.
419. Tenzel, William V.: Radiation therapy in carcinoma of the lung. *J. A. M. A.*, 117: 1778–1782, 1941.
420. Time Magazine, 76: 37, 1960.
421. Trincao, Mário: Cancro Primitivo do Pulmao. *Coimbra*, 1942.
422. Trump, John G., Clarence R. Moster, and Robert W. Cloud: Efficient deep tumor irradiation with roentgen rays of several million volts. *Am. J. Roentgen.*, 57: 703–710, 1947.
423. Turner, H. Midgley, and H. G. Grace: An investigation into cancer mortality among males in certain Sheffield trades. *J. Hyg.*, 38: 90–103, 1938.
424. Twort, J. M., and C. C. Twort: Disease in relation to carcinogenic agents among 60,000 experimental mice. *J. Path. and Bact.*, 35: 219–242, 1932.
425. Uhlig, Margarete: Uber den Schneeberger lungenkrebs. *Arch. f. path. Anat.*, 230: 76–98, 1921.
426. Umiker, W. O., M. S. DeWeese, and G. H. Lawrence: Diagnosis of lung cancer by bronchoscopic biopsy scalene lymph node biopsy, and cytologic smears. *Surg.*, 41: 705–713, 1957.
427. Vallee, B. L., D. D. Ulmer, and W. E. C. Wacker: Arsenic toxicology and biochemistry. *Arch. Ind. Health*, 21: 132–151, 1960.
428. Verga, Pietro, and Giuseppe Botteri: *Il Carcinoma Primitivo del Polmone*. L. Cappelli—editor, Bologna, 1931.
429. Vital Statistics of the United States and the United States Bureau of the Census Current Population Reports. *Population estimates*. Series P-25.
430. Vorwald, Arthur J., and John W. Karr: Pneumonconiosis and pulmonary carcinoma. *Am. J. Path.*, 14: 49–58, 1938.
431. Vorwald, A. J.: *Lecture to the Harvard School of Public Health*, Boston. (Unpublished—reference from Doll (85).)
432. Wallace, W. S., and Harold G. Jackson: Bronchogenic carcinoma: a resume and some newer concepts. *Tex. St. J. Med.*, 38: 605–612, 1943.
433. Wallner, Linden J.: Effects of smoking on the respiratory tract. *J. Internat. Coll. Surg.*, 27: 210–217, 1957.
434. Walshe, W. H.: *A Practical Treatise on Diseases of the Lungs*. James Walton, London, 1871.
435. Wassink, W. F.: Ontstaansvoorwaarden voor longkankee. *Neder. Tijds. v. Geneesk.*, 92: 3732, 1948. (Reference from Cutler (77).)
436. Watson, A. F., and E. Mellanby: Tar cancer in mice: (1) The technique of the comparative experiment. *Brit. J. Exper. Path.*, 11: 267–281, 1930.
437. Watson, T. A.: Supervoltage roentgen therapy in cancer of the lung. *Am. J. Roentgen.*, 75: 525–529, 1956.
438. Watson, William L.: Cancer of the Lung. Consideration of incidence and etiology. *N. Y. Med.*, 6: 15–18, 40, 1950.
439. Watson, William L., and Alexander J. Conte: Smoking and lung cancer. *Cancer*, 7: 245–249, 1954.
440. Wegelius, Carl: Changes in the lungs in 126 cases of asbestosis observed in Finland. *Acta Radiol.*, 28: 139–152, 1947.
441. Weller, Carl V.: The pathology of primary carcinoma of the lung. *Arch. Path.*, 7: 478–519, 1929.
442. Weller, Russell W.: Metaplasia of bronchial epithelium. *Am. J. Clin. Path.*, 23: 768–774, 1953.
443. Wells, H. Gideon, Maude Slye, and Harriet F. Holmes: The occurrence and

pathology of spontaneous carcinoma of the lung in mice. *Cancer Res.*, 1: 259–261, 1941.

444. Wheatley, B. M., C. T. Steed, E. W. Savage, J. H. King, E. W. Forster, H. J. Hodt, I. R. Jones, and D. W. Smithers: The two million volt van de Graaff generator installation designed for rotation therapy at the Royal Cancer Hospital. *Brit. J. Radiol.*, 26: 57–72, 1953.
445. Wilhelm, D. L.: The incidence of bronchogenic carcinoma. *Med. J. Australia*, 2: 284–292, 1951.
446. Willis, R. A.: *Pathology of Tumours*. The C. V. Mosby Company, St. Louis, 1948.
447. Winternitz, M. C., I. D. Wason, and F. P. McNamara: *The Pathology of Influenza*. Yale University Press, 1920.
448. World Health Organization Technical Report Series. *Epidemiology of Cancer of the Lung*. No. 192, Geneva, 1960.
449. Wyers, H.: Asbestosis. *Postgrad. Med. J.*, 25: 631–638, 1949.
450. Wynder, Ernest L., and Evarts A. Graham: Etiologic factors in bronchiogenic carcinoma with special reference to industrial exposures. Report of 857 proved cases. *Arch. Ind. Hyg. and Occup. Med.*, 4: 221–235, 1951.
451. Wynder, Ernest L., and Evarts A. Graham: Tobacco smoking as a possible etiologic factor in bronchiogenic carcinoma. A study of six hundred and eighty-four proved cases. *J. A. M. A.*, 143: 329–336, 1950.
452. Wynder, E. L.: Towards a solution of the tobacco-cancer problem. *Brit. Med. J.*, 1: 1–3, 1958.
453. Wynder, Ernest L., and Jerome Cornfield: Cancer of the lung in physicians. *N. Eng. J. Med.*, 248: 441–444, 1953.
454. Wynder, Ernest L., Irwin J. Bross, Jerome Cornfield, and W. E. O'Donnell: Lung cancer in women. A study of environmental factors. *N. Eng. J. Med.*, 255: 1111–1121, 1956.
455. Zacherl, Sylvester: Ueber das primäre Lungenkarzinom. *Wein. Klin. Wchnschr.*, 44: 967–970, 1931.

PROCEEDINGS OF THE TEXAS BRANCH OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

Abstracts of Some of the Papers Read at the Spring Meeting

MAY 5–6, 1961

TEXAS A&M COLLEGE, COLLEGE STATION

Stimulation by Vitamins K of the Growth of *Mycobacterium tuberculosis* var. *hominis* H37Rv.

James O. Norman and Robert P. Williams, Department of Microbiology, Baylor University College of Medicine, Houston, Texas.

Vitamin K stimulates the growth of *Mycobacterium tuberculosis* var. *hominis* H37Rv when incorporated into Proskauer and Beck medium. A series of vitamins K_1 and K_2, and vitamin K-like compounds were tested. The most active compound was vitamin $K_1(20)$. At concentrations between 16 to 20 *ug.* per ml. growth was measurable by turbidimetric means within 48 to 72 hr., and was markedly increased at 168 hr. incubation. Control cultures showed no growth. The data suggested that a quinone or naphthoquinone structure, an unsaturated side chain at position 3 in the quinone ring, and the substituted group on the 3rd carbon atom of the side chain were important for stimulation of growth. K_1 diacetate, menadione, and lomatiol did not stimulate growth. The length of the side chain also was important since $K_1(25)$, $K_2(30)$ and $K_2(50)$ were completely inactive.

Amino Acid Requirements for Temperature Induced Pigmentation in *Serratia marcescens*.

Cora Lee Gott and Robert P. Williams, Department of Microbiology, Baylor University College of Medicine, Houston, Texas.

Employing the temperature induced pigmentation system described by us previously, we have investigated the amino acid requirements for pigment synthesis. Fractionation of casein hydrolysate into constituent amino acids demonstrated that only a few were required. When added singly to the non-proliferating cell suspensions, these amino acids caused pigment synthesis. The increase in pigment formation was similar to that of growing cultures. Proline and alanine were the most efficient, followed, in order, by ornithine, glutamic, and aspartic acids. L-proline was active, but the D form appeared to be inhibitory. Both D and L-alanine caused pigment formation, the latter being best. None of 18 other amino acids tested produced detectable pigment. A schema of pigment synthesis was proposed involving the known pathway of proline synthesis from glutamic acid or ornithine through pyrroline carboxylic acid. Aspartic acid could fit into the schema by transamination. The place of alanine in pigment synthesis is not known. The data indicate that

pigment arises from amino acids and involves a previously unknown pathway of pyrrole synthesis.

Preparation of Spheroplasts from *Vibrio comma*. B. R. Chatterjee and Robert P. Williams, Department of Microbiology, Baylor University College of Medicine, Houston, Texas.

Preparation of spheroplasts from vibrios has not been successful. We have prepared spheroplasts from *Vibrio comma* by lysozyme treatment combined with freezing and thawing. Lysozyme was added to 4 hr. broth cultures of vibrios supplemented with 15% sucrose. Two minutes after addition of lysozyme, the samples were frozen by immersion in a dry ice-acetone bath. After freezing, the samples were thawed at 37° C. in a shaker. Spheroplasts formed in 1–2 hr. No cell wall was seen under phase microscopy. The spheroplasts were osmotically sensitive and older ones contained granules. Optimum lysozyme concentration of 50 *ug*/ml transformed 70–85% of cells into spheroplasts. Higher concentrations of lysozyme caused cell lysis. Addition of 3% glycine instead of lysozyme brought about spherical transformation without freezing and thawing. The glycine treated cells were osmotically insensitive, actively motile, and morphologically different from lysozyme spheroplasts.

Transductional Studies in A Polylysogenic Strain of *Staphylococcus Aureus*. C. E. Dowell and E. D. Rosenblum, Department of Microbiology, University of Texas Southwestern Medical School, Dallas, Texas.

After treatment of a sensitive indicator strain with a lysate from a lysogenic strain of *Staphylococcus aureus*, a strain was isolated which was triply lysogenic. The carried phages differed in serology, plaque morphology, and host range. Tests carried out using a liquid culture method and appropriate indicator strains showed that one of the phages could transduce streptomycin resistance and novobiocin resistance, while the other two could not. Using this phage, the mode of expression of these two antibiotic markers was studied.

Infectivity of Adsorbed Staphylococcal Phage. Mary Beth Laughlin and E. D. Rosenblum. Department of Microbiology, University of Texas Southwestern Medical School, Dallas, Texas.

The titer of infectious phage present in a lysogenic culture varies according to method of assay. Supernatants of centrifuged cultures (free phage) gave lower titers than whole cultures plated directly on antibiotic media with antibiotic resistant indicator strains (plaque forming units-PFU).

Preliminary experiments ruled out induction of lysis by the antibiotic as cause for the discrepancy. During growth of lysogenic strains, PFU's and free phage are equal and rise until readsorption by the cells is appreciable, the PFU titer then becoming higher. This suggests that phage adsorbed by homologous cells are still infectious. Chloroform and Waring Blender treat-

ments showed that adsorbed phage cannot readily be removed from the cell.

Studies of phage adsorbed to either a viable or a heat treated resistant strain indicated that adsorption of heterologous phage and its inactivation occur almost simultaneously. Lysogenic cells, however, have a limited capacity for adsorption of homologous phage from an external source. Immunity of lysogenic cells to homologous phage is distinguished from immunity to heterologous phage in coagulase positive Staphylococci in at least two properties—less efficient adsorption, and infectivity of the adsorbed phage.

Preliminary Serological Studies of Marine Actinomycetes.*

R. K. Guthrie, A. W. Roach, J. K. Ferguson and C. Mitchell. North Texas State College, Denton, Texas.

A short injection schedule immunizing procedure using sonically treated aerobic marine actinomycete antigens emulsified in paraffin oil and lanolin, has produced a satisfactory antiserum for study of serological relationships of a group of nineteen of these organisms isolated by this laboratory from marine environments. Agar diffusion precipitation was used to determine serological reactions. The most intense band of precipitation in homologous reactions was termed the major antigen reaction; less intense bands were termed minor antigen reactions. Using three specific antisera, fourteen isolates were separated into five groups. Five isolates did not cross react with any antiserum. Future study will include additional marine isolates, and known cultures, as studied by these and additional antisera.

A Mineral Salts-Cholesterol Agar for Culturing and Distinguishing Cholesterol-Using Soil Actinomycetes.

Harold Lewis, James R. Davis and G. E. Peterson, Department of Biology, University of Houston, Houston, Texas.

It is known that certain soil microorganisms can utilize cholesterol as a sole carbon source. Previous studies have been frustrated by: (1) the long incubation period (several weeks) required for the breakdown of the sterol in enrichment broths, and (2) the lack of a solid growth medium containing cholesterol as a sole carbon source.

This study describes the isolation of rapid as well as slow cholesterol users from soil microflora, a homogenized cholesterol agar for distinguishing slow from fast cholesterol users, the variable morphology of cholesterol-using soil microorganisms when grown on various media, and the identity of these microorganisms with the actinomycetes.

When enrichment cultures were streaked on agar plates containing "normally" suspended cholesterol as a sole carbon source, the sterol was poorly dispersed and little or no growth resulted. However, when a Waring Blendor® was used to disperse the sterol in molten agar, homogeneous plates were obtained which supported good growth of cholesterol users. Two types of growth were observed on homogenized plates—growth with a cleared zone

* Work supported by NIH Grant-in-aid E-2560.

around the colony and growth with no clearing. In broth cultures, the former used cholesterol rapidly and the latter slowly. The cleared zones of fast cholesterol users seemed to reflect a low, soluble concentration of cholesterol rather than the action of an extracellular enzyme. Actinomycetes with great variations in staining and colonial and cellular morphology were observed when these microorganisms were grown on various media. These variations may account for the conflicting conclusions of other workers concerning the taxonomic position of cholesterol-degrading soil microorganisms.

Studies on the Incorporation of Uridine into the Ribonucleic Acid Components of Irradiated Bacteria.

E. W. Frampton. Section of Microbiology, Department of Biology, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston.

Measurements have been made of the influence of X-irradiation (90K r dose) on the ability of log phase cells of *Escherichia coli* strain B/r to incorporate labeled uridine into various RNA components. Fractions designated P_1 (20,000 x *g* for 15 min), P_2 (144,000 x *g* for 120 min), and S (the remaining supernatant material) were prepared from cells ground with alumina and from cells disrupted by passage through a French pressure cell. By means of sucrose density gradient centrifugation, additional fractionations were also carried out on resuspended P_2 fractions and concentrated S fractions.

Total labeling of RNA was lower in all fractions obtained from irradiated cells. However, a statistical analysis of the slopes of the linear portion of the incorporation curves (specific activity of RNA vs. time) showed a depressed rate of incorporation in the S fraction obtained from irradiated cells disrupted in the French cell and in the P_1 fraction from alumina-ground cells.

Sucrose density gradient analyses of P_2 fractions (ribosomes) and S fractions (soluble RNA, 4-8S RNA, and small ribosomes) from cells disrupted in a French cell suggest that the labeling of a RNA component in the S fraction may be preferentially suppressed by irradiation.

TECHNICAL REPORTS

SOME COMPARATIVE EFFECTS OF SMOKED PAPER, TOBACCO AND CIGARETTES ON CHROMOSOMES IN VITRO[a]

AKIO AWA,[b] YASUSHI OHNUKI,[b] AND C. M. POMERAT

Introduction

Current interest in the relation of tobacco smoke as a cause of lung cancer is reflected in several reviews (*e.g.*, 3, 19, 20). Special attention has been directed to the production of lung and skin malignancies in animals treated with tobacco tar or condensates (2, 4, 5, 15, 16). Histological and cytological studies have also been done on the effects of cigarette smoke and tobacco condensates upon cells *in vivo* or *in vitro* (6, 7, 8, 13).

The aim of the present study was to extend basic knowledge regarding the effect of cigarette smoke and its constituents on cells of an established human lung strain cultivated *in vitro*, with special attention to mitotic and chromosomal irregularities.

Materials and Methods

Cells of human foetal lung (HL-C strain) established by Nakanishi *et al.* (13) were cultivated either in T-flasks or in Rose chambers. Initial seeding in each experimental flask or chamber was adjusted to form a monolayer covering the floor of the container by the 6th day. Cells were then exposed to the smoke obtained from (A) whole cigarette, (B) tobacco without paper, and (C) "Riz la 7" paper alone. These materials were burned in glass tubes of the same length and internal diameter as the corresponding cigarette. After removal of the nutrient medium, smoke from these test materials, collected by sucking with the use of a 50 ml or a 100 ml syringe fitted to the glass tube, was introduced directly into the container in which the cells were growing. After 5 to 10 seconds the smoke was removed thoroughly with the aid of a suction pump from the culture vessels. Fresh Eagle's medium containing 10% inactivated horse serum was then added. The volume of smoke used in each experiment was 1.5, 30, and 70 ml for Rose chambers, T-30, and T-60 flasks, respectively. Approximately 25 times the weight of paper was employed as that used for the corresponding cigarette. Chromosome spreads were made 24 hours after treatment using the hypotonic sodium citrate-

[a] From the Division of Cellular Biology, Pasadena Foundation for Medical Research, Pasadena, California. This investigation was supported in part by a research grant (RG-8000) from the Division of Special Health Services, Public Health Service, National Institutes of Health. Submitted for publication May 1, 1961.

[b] Fellows of the Tobacco Industry Research Committee. Permanent address: Zoological Institute, Hokkaido University, Sapporo, Japan.

acetic orcein, squash technique described in a previous report (14), with the exception that no colchicine was employed.

Observations on chromosomes were performed with the use of dark phase contrast microscopy employing 100x objectives and 8x or 12x oculars. Further morphological observations on living cells were made on cultures in Rose chambers, using bright phase contrast (43x objectives, 10x eyepieces). They were then fixed with absolute methanol and stained with the May-Grunwald-Giemsa technique.

The percentage of cells in mitosis in each experiment was determined on the basis of a minimum of 2000 cells. Further assessment of irregular mitoses was made by counting 200 dividing cells for each experimental condition by means of random sampling.

Results

(1) GENERAL OBSERVATIONS

In control preparations, the HL-C cells were morphologically of the epithelial type with several well-defined nucleoli. All cells showed active cytoplasmic movement characterized by fine amoeboid protrusions. These are shown in the living state in Figure 1, and after staining (Fig. 5). The strain grew rapidly, requiring frequent subculture. A peculiar feature, noted during this process, was an unsual difficulty in breaking up cell aggregates. Even after agitation several times with a syringe, large cell colonies remained intact.

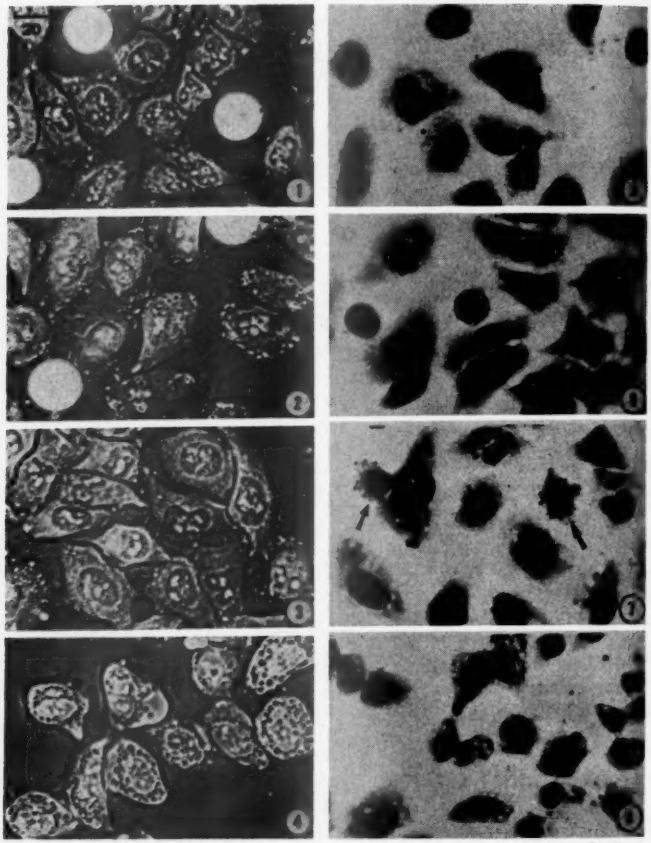
Cells exposed to the smoke collected from whole cigarettes (Figs. 2 and 6) and from tobacco alone (Figs. 3 and 7), showed little evidence of injury. Only a few cells treated with tobacco smoke were damaged as evidenced by blebbing of the cytoplasm, together with slightly pycnotic nuclei in May-Grunwald-Giemsa preparations (Fig. 7, arrows).

In contrast, remarkable degenerative changes were found both in the cytoplasm and nucleus of cells exposed to paper smoke. Within 6 hours after treatment, some cells displayed vacuolization and blebbing of the cytoplasm. By 24 hours, these changes became more prominent, and observations with the phase contrast microscopy showed that every cell was severely vacuolated (Fig. 4). In addition, stained preparations revealed that the nuclei were severely pycnotic, so that nucleoli were obscure (Fig. 8).

(2) EFFECT OF SMOKE ON DIVIDING CELLS

(a) *Mitotic Counts and Injuries*

In control cultures on the 7th day after incubation, 9 percent



The scale of magnification is given in the upper left area of each figure representing cellular elements.

FIGS. 1–4. Photomicrographs of HL-C cells, with phase contrast microscopy, exposed to the smoke. 24 hours after exposure.

FIG. 1. Control. White rounded cells are in mitosis.

FIG. 2. Cells exposed to whole cigarette smoke.

FIG. 3. Cells exposed to tobacco smoke.

FIG. 4. Cells exposed to paper smoke, showing a number of vacuoles in the cytoplasm.

FIGS. 5–8. HL-C cells exposed to the smoke 24 hours after treatment. Stained by May-Grunwald-Giemsa.

FIG. 5. Control.

FIG. 6. Smoke from whole cigarettes.

FIG. 7. Smoke from tobacco. Arrows indicate damaged cells.

FIG. 8. Smoke from paper. Vacuolization of the cytoplasm with pycnotic nuclei.

of cells (Fig. 9) were in mitosis, there being a variation from 7 to 10 percent in various samples. Of these, 18.2 percent were abnormal (Fig. 10).

Treatment with smoke of either whole cigarettes or tobacco alone led to an increase in the rate of mitotic irregularities and a decrease in the mitotic index. Mitotic indices were 4.2 and 3.1 percent, with a variation ranging from 3 to 6 percent and from 2.5 to 6 percent, respectively. The frequency of abnormal mitoses induced by tobacco smoke was somewhat higher (34.5%) than for whole cigarettes (23.0%).

Treatment with paper alone led to a considerable depression of the mitotic index; *viz.*, 2 to 3 percent, and many abnormal cell divisions (Figs. 9 and 10). Most metaphase figures were affected. In one experiment, 87.5 percent of all dividing cells were morphologically abnormal.

(b) *Chromosomal Abnormalities*

As a baseline study chromosome counting was carried out in squash preparations of untreated HL-C cells. In a recent sample (March, 1961), 70 percent of metaphases observed had 68 chromosomes which seemed to be the stemline number (Table 1 and Fig. 12). The fluctuation around the modal value was rather narrow, ranging from 65 to 77, and about 10 percent of the cells were polyploids.

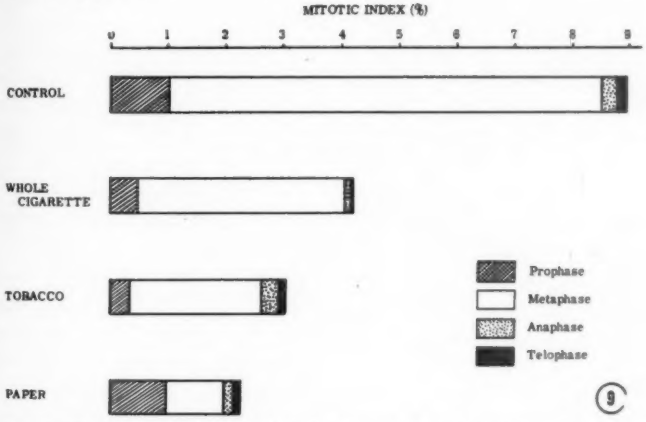


FIG. 9. Histograms of the mitotic index in control and smoke-exposed HL-C cells. 24 hours after exposure.

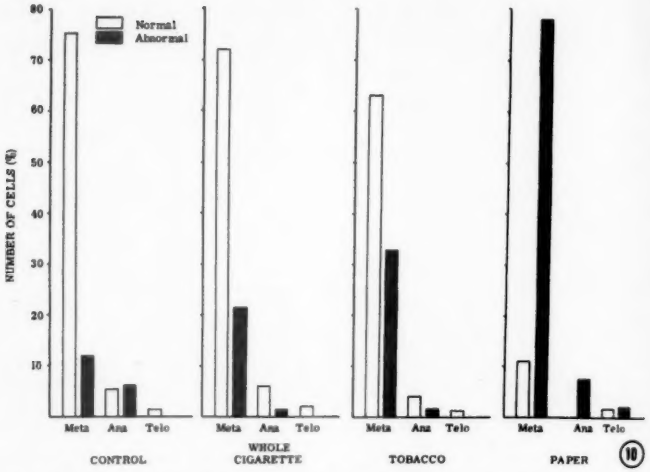


FIG. 10. Distribution of normal and abnormal cells in each mitotic phase. 24 hours after treatment with smoke.

Mitotic irregularities found in cells exposed to whole cigarette and tobacco smoke were mainly characterized by condensation or contraction of the chromosmes at metaphase (Figs. 13, 14, and 15). This abnormality occurred in 10 and 26 percent of the cells treated with whole cigarette and tobacco smoke, respectively (Fig. 11), resembling the injury produced by colchicine or podophyllin and its derivatives (12, 18). The degree of contraction in chromosomes ranged from ½ to ¾ of the length in control cells. Some elements showed chromosome stickiness. However, the occurrence of this anomaly was not as significant as that of the controls.

In the paper smoke series there was a different abnormality. Figure 11 and Figures 16 through 19 show a remarkable degree

TABLE 1

Distribution of chromosome numbers in human lung strain (HL-C) cells after 3 years of serial passages (March, 1961)

Chromosome number	65	66	67	68	—	74	—	77	Total
Number of cells observed	1 (5%)		3 (15%)	14 (70%)	—	1 (5%)	—	1 (5%)	20

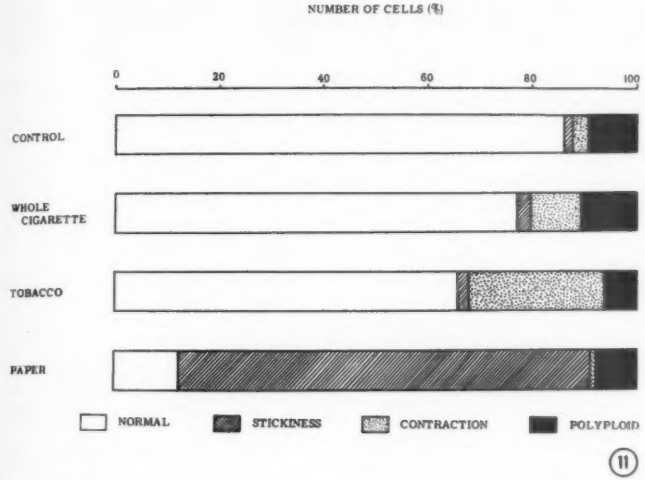


FIG. 11. Distribution of abnormal metaphases exposed to various types of smoke 24 hours after exposure.

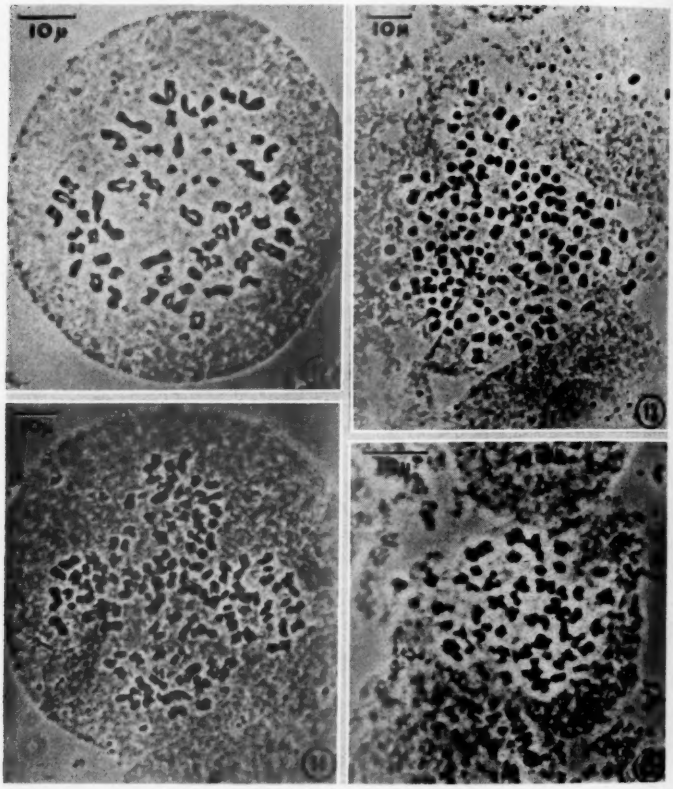
of chromosome stickiness which was a constant feature in all the paper smoke experiments. In addition, some cells seemed to show chromosome translocations (Figs. 16 and 17).

Anaphase and telophase anomalies, namely bridging and lagging chromosmes, were found in equal numbers in untreated cultures and in those exposed to whole cigarette and tobacco smoke. Treatment with paper alone, however, led to a considerable increase in these abnormalities.

Discussion

Leuchtenberger *et al.* (6, 7, 8) have reported a series of correlated cytological, histological, and cytochemical studies of lung tissues in mice exposed to cigarette smoke. In these articles, they demonstrated that bronchitis, basal-cell hyperplasia and a typical basal-cell hyperplasia were found in the majority of animals while a few developed squamous metaplasia. The DNA content correlated with these events.

As suggested by Nakanishi *et al.* (13), little attention has been directed to studies based on cytological and chromosomal observations. Certain cigarette tar fractions were shown to pro-



FIGURES 12 to 15. Photomicrographs of the chromosomes in control and smoke-treated HL-C cells. 24 hours after exposure.

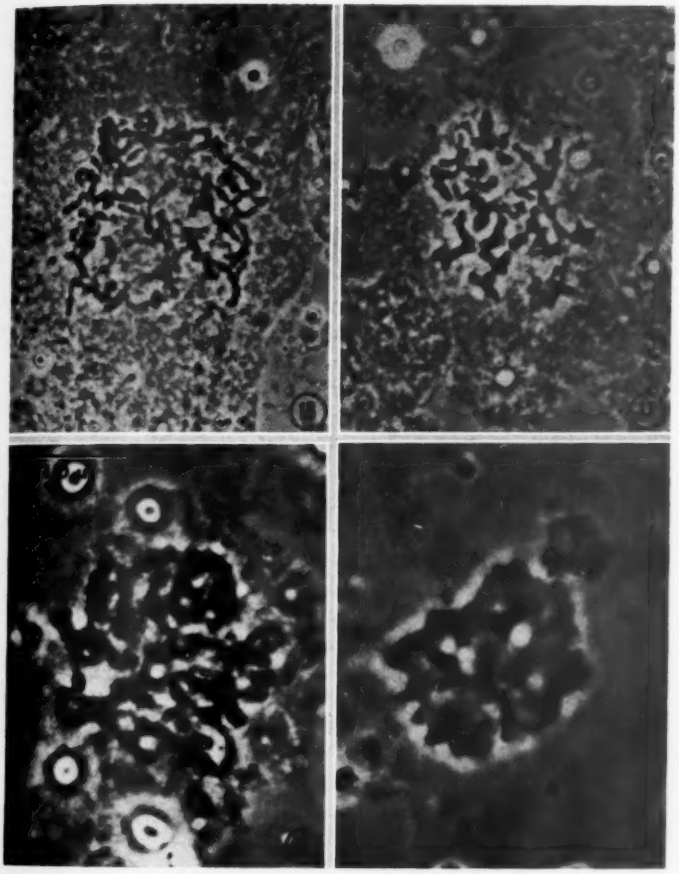
FIG. 12. Untreated control cell. 68 chromosomes.

FIG. 13. Metaphase treated with whole cigarette smoke. Polyploid together with slightly pycnotic chromosomes.

FIG. 14 and 15. Metaphases exposed to tobacco smoke. FIG. 14. Polyploid. FIG. 15. Metaphase showing notable contraction of chromosomes.

duce injury to mitochondria in human lung cells *in vitro* (Nakanishi *et al.* 13). Further long-term treatment with such condensates led to shifts in the stem line chromosome number of a corresponding cell line (HL-S) which was obviously different from that of the untreated controls (HL-C).

Somewhat apart from this viewpoint, the main purpose of the present study was to inquire whether degenerative changes could



FIGURES 16 to 19. Photomicrographs of HL-C cells exposed to paper smoke showing various degrees of chromosome stickiness at metaphase. 24 hours after treatment.

be produced by direct exposure to the smoke collected from each of whole cigarettes in contrast to tobacco and paper alone.

Under the experimental conditions employed, paper smoke was shown to be notably toxic. Vacuolization of the cytoplasm together with pycnotic nuclei was seen in most cells 24 hours following exposure to this factor. In contrast, the majority of cells

exposed to smoke from the other two sources did not exhibit any appreciable degenerative change. From mitotic cell counts each type of smoke was shown to induce a considerable decrease in the rate of division and a significant increase in the number of abnormal mitoses.

Of a variety of mitotic irregularities in cells treated with the smoke from whole cigarettes and from tobacco alone, contraction of chromosomes at metaphase was notable. In contrast, paper smoke produced chromosome stickiness in most metaphases. Consequently, sticky-bridges were frequently observed in cells at anaphase and telophase.

In order to interpret the marked difference between whole cigarette smoke and that of its constituents, the following two possibilities might be considered. One is that paper smoke might have a certain fraction or fractions which are apparently noxious enough to produce chromosome stickiness, while smokes from whole cigarette and from tobacco alone contain little or no such substance but other products which might cause different mitotic abnormalities. Radiation and radiomimetic chemicals have been shown to act as mitotic poisons which induce chromosome stickiness (1). The paper smoke effect might fall in this category. Another possibility lies in the difference in the concentrations and temperature of each smoke when they are burned and collected. Care was taken to achieve uniformity in this respect but without complete quantitative controls. Chromosome stickiness which can be readily recorded may prove valuable for future studies not only in the assessment of injury from cigarette and its constituents but other noxious products in the atmosphere.

The modal chromosome number of the HL-C was reported as 77 by Nakanishi *et al.* (13). As a by-product of this study it was shown that the modal value, found in 70 percent of the cells observed had changed to 68 after 3 years of successive passages since its establishment. In addition, there was evidence that a peculiar feature of this cell line was the formation of more firmly adherent cell colonies. Such a morphological change might be correlated with the shift in the stemline chromosome number. In a number of investigations (cf. 9, 10, 11, 13, 17) it has been pointed out that the stemline cell which is characterized by having a definite idiogram and chromosome number may undergo a

change in pattern under *in vitro* conditions. Of special interest was the shift in the modal value toward a lower chromosome number.

Summary

Mitotic and chromosomal analyses were carried out in the HL-C strain cells exposed directly to the smoke collected from (A) whole cigarettes, (B) tobacco alone, and (C) paper alone. Morphological observations revealed that paper smoke induced severe vacuolization of the cytoplasm together with pycnotic nuclei in most cells. In contrast, little evidence of cellular damage was shown by exposure of smoke from whole cigarette and from tobacco alone.

There was a decrease in the rate of the mitotic index and a considerable increase in the number of abnormal divisions as a result of the treatment with each substance, particularly with paper smoke. Contraction of chromosomes was noticed in some metaphases exposed to smoke from whole cigarettes and from tobacco. In contrast, paper smoke produced a high incidence of chromosome stickiness.

These mitotic abnormalities and the shift in the modal chromosome number of the HL-C strain are briefly discussed.

Acknowledgments

Special thanks are due to Messrs. C. G. Lefeber, R. Olson and W. Huff for their aid in the photographic work. Grateful acknowledgment is also extended to Mr. C. W. Raiborn, Jr., for the management of the culture preparations. The authors are indebted to Dr. H. G. Richmond for his valuable encouragement.

REFERENCES

1. Biesele, J. J.: *Mitotic poisons and the cancer problem.* Elsevier Publ. Co., Houston, 1958.
2. Bock, F. G., and M. E. Moore: Carcinogenic activity of cigarette smoke condensate. I. Effect of trauma and remote X-irradiation. *J. Nat. Cancer Inst.* 22: 401–411, 1959.
3. Cornfield, J., W. Haenszel, E. C. Hammond, A. M. Lilienfeld, M. B. Shimkin, and E. L. Wynder: Smoking and lung cancer. Recent evidence and a discussion of some questions. *J. Nat. Cancer Inst.* 22: 173–203, 1959.
4. Engelbreth-Holm, J., and J. Ahlmann: Production of carcinoma in ST/Eh mice with cigarette tar. *Acta. path. et microbiol. Scandinav.* 41: 267–272, 1957.

5. Gellhorn, A.: The cocarcinogenic activity of cigarette tobacco tar. *Cancer Research* 18: 510–518, 1958.

6. Leuchtenberger, C., R. Leuchtenberger, and P. F. Doolin: A correlated histological, cytological, and cytochemical study of the tracheobronchial tree and lungs of mice exposed to cigarette smoke. I. Bronchitis with atypical epithelial changes in mice exposed to cigarette smoke. *Cancer* 11: 490–506, 1958.

7. Leuchtenberger, C., R. Leuchtenberger, W. Zebrun, and P. Shaffer: Ditto. II. Varying responses of major bronchi to cigarette smoke, absence of bronchogenic carcinoma after prolonged exposure, and disappearance of bronchial lesions after cessation of exposure. *Cancer* 13: 721–732, 1960.

8. Leuchtenberger, R., C. Leuchtenberger, W. Zebrun, and P. Shaffer. Ditto. III. Unaltered incidence of grossly visible adenomatous lung tumors in female CFl mice after prolonged exposure to cigarette smoke. *Cancer* 13: 956–958, 1960.

9. Levan, A.: *Relation of chromosome status to the origin and progression of tumors: The evidence of chromosome numbers. In* Genetics and Cancer, Univ. of Texas Press, Austin: 151–182, 1959.

10. Makino, S.: The chromosome cytology of the ascites tumors of rats, with special reference to the concept of the stemline cell. *Intern. Rev. Cytol.* 6: 25–84, 1957.

11. Makino, S., and M. Sasaki: Cytological studies of tumors: XXI. A comparative ideogram study of the Yoshida sarcoma and its subline derivatives. *J. Nat. Cancer Inst.* 20: 465–487, 1958.

12. Makino, S., and T. Tanaka: The cytological effect of chemicals on ascites sarcomas. I. Partial damage of tumor cells by podophyllin, followed by temporary regression and prolongation of life of tumor-bearing rats. *J. Nat. Cancer Inst.* 13: 1185–1199, 1953.

13. Nakanishi, Y. H., M. Mizutani, and C. M. Pomerat: Smoke condensates on lung cells in tissue culture with special reference to chromosomal changes. *Texas Rep. Biol. Med.* 17: 542–590. 1959.

14. Ohnuki, Y., A. Awa, and C. M. Pomerat: Chromosomal studies on irradiated leucocytes *in vitro*. *Ann. N. Y. Acad. Sci.* (in press).

15. Orris, L., B. L. Van Duuren, A. I. Kosak, N. Nelson, and F. L. Schmitt: The carcinogenicity for mouse skin and the aromatic hydrocarbon content of cigarette-smoke condensate. *J. Nat. Cancer Inst.* 21: 557–562, 1958.

16. Reddy, D. G., D. B. Reddy, and P. R. Rao: Experimental production of cancer with tobacco tar and heat. *Cancer* 13: 263–269, 1960.

17. Sasaki, M.: Cytological studies of tumors. XXVIII. Some experimental studies in two sublines of the Yoshida sarcoma, with special consideration of the derivation of sublines. *Cytologia* 25: 69–85, 1960.

18. Tanaka, T., K. Kano, A. Tonomura, T. A. Okada, and M. Umetani: The cytological effect of chemicals on ascites sarcomas. III. Damage induced by podophyllotoxin, alpha-peltatin, beta-peltatin, and quercetin. *Gann* 46: 15–26, 1955.

19. Wynder, E. L., and D. Hoffman: *Tobacco smoking as a cause of cancer. In* Cancer Progress Volume 1960 (Ed. R. W. Raven), Butterworth, England: 77–80, 1960.

20. Wynder, E. L., and G. Wright: A study of tobacco carcinogenesis. I. The primary fractions. *Cancer* 10: 255–271, 1957.

THE EFFECT OF GUANIDINE:

(1) On Experimental Poliomyelitis Induced by Oral Administration of Virus to Cynomolgus Monkeys

*(2) On Naturally Occurring Enteroviruses of Cynomolgus Monkeys**

JULIO G. BARRERA-ORO AND JOSEPH L. MELNICK

Guanidine is one of the rare compounds which inhibit the growth of poliovirus in tissue culture (6). Rightsel *et al.* (6) carried their experiments over into monkeys and observed suggestive therapeutic activity in animals infected by the intramuscular route.

Because we readily confirmed the antiviral effect of guanidine in tissue culture (1), we were led to explore the effects of the drug in cynomolgus monkeys which can be infected with poliovirus by the natural oral route. As young monkeys frequently excrete ECMO (Enteric Cytopathogenic Monkey Orphan) viruses in their feces (2,3), we also had an opportunity to determine whether guanidine might suppress excretion of these primate enteroviruses, particularly in view of the inhibiting action reported for guanidine on non-polio enteroviruses (6).

Materials and Methods

Monkeys. 36 young *Macaca cynomolgus* were used; they ranged in weight from 1.1 to 2.9 Kg (average 1.7 Kg). They were pretested for neutralizing antibodies to poliovirus Type 1; all were found to be negative.

Treatment with guanidine-HCl. The guanidine dosage was based on the prior work of Rightsel *et al.* (6) who determined the limiting amounts of the drug which could be tolerated by rhesus monkeys. Because of its rapid excretion in the urine (6), guanidine was administered three times daily by stomach tube. The course was begun three days before the animals were challenged with poliovirus, and was continued for 21 days after the virus feeding, or until the animal developed serious disease. The animals were divided into three groups as follows:

a) 12 animals received a "low" guanidine dosage: 60 mg per Kg of body

* From the Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas. Aided by a Research Grant from Parke, Davis & Company. Submitted for publication April 4, 1961.

weight daily, administered by stomach tube:

8 a.m.	30 mg per Kg
3 p.m.	15 mg per Kg
10 p.m.	15 mg per Kg

b) 7 animals received high guanidine dosage: 80 mg per Kg of body weight daily, administered by stomach tube:

8 a.m.	40 mg per Kg
3 p.m.	20 mg per Kg
10 p.m.	20 mg per Kg

Initially, monkeys in the high dosage group received a total of 120 mg per Kg body weight daily (60, 30 and 30 mg), but after three days 5 of the original 12 in this group died from guanidine toxicity, and the dosage was therefore reduced for the survivors.

c) 12 control animals received no treatment with guanidine, but were challenged orally with poliovirus in parallel with the test animals.

The challenge poliovirus. Three strains of Type 1 poliovirus were administered by feeding. Their titers were as follows:

Houston (Bender) strain	$10^{8.5}$ TCD_{50} per ml
Israel—1958 epidemic strain	$10^{8.2}$ TCD_{50} per ml
Mahoney strain	$10^{7.5}$ TCD_{50} per ml

The material was prepared by mixing 20 ml of undiluted infectious tissue culture fluid with canned banana baby food, and letting the monkeys eat the mixture. This they did avidly except for one monkey (# 775) which ate only part of the preparation and therefore was given 10 ml of undiluted tissue culture fluid by stomach tube. Initially, four monkeys at each guanidine dosage, and four controls, were to be challenged with each of the three poliovirus strains. With the loss of 5 monkeys from the high guanidine group at the outset, only 7 of this group were available. Of these, 2 received the Israel strain, 3 the Houston strain, and 2 Mahoney.

Observations. The monkeys were examined daily for development of clinical signs of poliomyelitis and four obvious drug reactions. The observations were continued for up to 38 days following the poliovirus feeding, unless the animal died or was sacrificed because of the severity of its disease. Multiple sections of the spinal cord and brain stem were taken for histological examination.*

Virus Excretion. Fecal samples were taken as follows:

a) nine days before guanidine administration began,
b) on the day guanidine was first given,
c) after 3 days of treatment, i.e., just prior to virus challenge,
d) one week after virus challenge,
e) two weeks after virus challenge.

Fecal specimens were tested for the presence of enteroviruses, using monkey kidney cultures, by methods already described (4). The viruses isolated were not typed.

Viremia. Blood specimens were drawn on days 3, 5, 7, and 10 following

* We are grateful to Dr. R. J. McAlpine for preparation and examination of histological specimens.

virus feeding. The sera were tested for the presence of poliovirus, and positive specimens were titrated by the cytopathogenic endpoint method in monkey kidney cultures (4).

Poliovirus antibody. In addition to the pre-challenge determinations to ascertain that the monkeys were free of polio antibodies, the animals surviving the observation period were bled at the time of sacrifice, and the sera were tested for the development of neutralizing antibodies to Type 1 poliovirus during the experimental period. The metabolic inhibition (pH) test in panels was used (5, 7).

Virus in CNS. Isolation of poliovirus was attempted from the spinal cords of some animals, again using monkey kidney cultures.

Results

In Table 1, clinical and histopathological findings, the presence or absence of viremia, and antibody titrations are shown for the monkeys receiving each of the three challenge poliovirus strains, with low and high guanidine dosages and for control monkeys which did not receive the drug.

Animals which died of guanidine toxicity but which were free of poliomyelitis lesions (#755, 763, and 766) are included in this table, but have been omitted in making up the summary of clinical responses (top of Table 3). In all other animals, the clinical signs of poliomyelitis and the finding of poliomyelitis lesions in the central nervous system occurred in parallel, except in #765, which had poliomyelitis lesions in the CNS, virus in CNS, and viremia, but whose clinical picture was not typical of poliomyelitis but may have been due to guanidine toxicity superimposed on the virus disease.

Where virus isolation was attempted from CNS, the results also paralleled the clinical and histopathologic findings.

No viremia could be detected in two animals, #762 and 787, although they developed clinical signs and CNS lesions of poliomyelitis. The opposite situation was found in two other monkeys (#773 and 782), in which viremia was found but clinical signs and poliomyelitis lesions were absent.

In Table 2 are shown the detailed results of tests for viremia in sera taken 3, 5, 7, and 10 days after challenge virus was fed. Poliovirus titers in the serum were generally low; they ranged from 16 to 60,000 TCD_{50} per ml of serum.

In the 2 monkeys receiving high guanidine dosage and challenged with the Israel strain of poliovirus, only 1 had viremia,

TABLE 1

Responses of Cynomolgus Monkeys to Oral Challenge with Type 1 Poliovirus as Related to Treatment with Guanidine. Clinical Signs, Lesions and Recovery of Virus in CNS, and Viremia

Challenge Virus	Guanidine Dosage	Monkey No.	Clinical Signs	Polio Lesions in CNS	Poliovirus in CNS	Polio Viremia	Polio Antibody Titer
		749	12,WL; 13,pBL; 20,pBL←; 28, WBL (36)	+	..	+	>1024
	LOW	750	9,WBA; 11,PBL; 16,WBA←; 20,pBL←; 28,WBL (36)	+	..	+	1024
		751	15,PLL; 17,PBL (29)	+	..	+	512
		756	6,PRA; 7,PBA&L, Tr. (7)	+	..	+	...
ISRAEL		762	19,PBL; 20,PBA (22)	+	+	0	...
1958	HIGH	763*	16, D	0	0	+	...
		775	O.K. (36)	0	..	0	8
		776	11,PBL; 12,WA (29)	+	..	+	512
	CONTROLS	777	O.K. (36)	0	..	0	0
		778	O.K. (36)	0	..	0	16
		752	6,W; 8,PBL,pBA (8)	+	+	+	...
		753	9,pBL; 10,PBA&L (11)	+	+	+	...
	LOW	754	14,pLL→; 16,pBL (36)	+	..	+	1024
		755*	9,W; 11,D	0	0	0	...
HOUSTON		765	7,W; 8, Spastic paresis, ataxia (8)	+	+	+	...
(Bender)	HIGH	766*	6,D	0	..	0	...
		767	O.K. (36)	0	..	0	0
		773	O.K. (38)	0	..	+	64
		780	O.K. (36)	0	..	0	12
	CONTROLS	781	14,WBL; 21,WBL←; 24,O.K. (38)	+	..	+	64
		782	O.K. (38)	0	..	+	1024

			24,O.K. (38)	+	..	+	64
		782	O.K. (38)	0	..	+	1024

Challenge Virus	Guanidine Dosage	Monkey No.	Clinical Signs	Polio Lesions in CNS	Poliovirus in CNS	Polio Viremia	Polio Antibody Titer
		757	5,W; 6,PRA; Conv., D	+	..	+	...
		758	11,PLA,WLL,Tr.;				
	LOW		13,PLA,WBL (14)	+	..	+	...
		759	9,pLA→; 10,PBA&L (12)	+	+	+	...
		760	6,pBAL→; 7,Conv., D	+	..	+	...
		768	11,PLA; 12,pBL,PBA;				
	HIGH		13,BBA&L (13)	+	..	+	...
MAHONEY		779	O.K. (29)	0	..	0	0
		784	10,PRA; 11,PBA&L (11)	+	..	+	...
		785	9,WLL; 10,PBL,WBA;				
			11,PBL&A (14)	+	..	+	...
	CONTROLS	786	14,WBL; 15,PBA&L (17)	+	..	+	...
		787	8,WBL; 10,pBL;				
			11,PBL,WBA (14)	+	..	0	...

A = arm
L = leg
LA = left arm
LL = left leg
BL = both legs

P = paralysis
p = paresis
W = weak
Conv. = convulsions
Tr = tremors

D = died
← = illness began subsiding
O.K. = no signs of disease
+ = present
0 = absent

The numbers preceding any combination of the above indicate the day after virus challenge at which a given sign first appeared; unless otherwise indicated, this sign persisted without subsiding until day animal was sacrificed.

* Cause of death difficult to establish in monkeys #755, 763, 766. These animals have been omitted from summary Table 3. They probably died of guanidine toxicity.

TABLE 2

Poliomyelitis Viremia in Cynomolgus Monkeys Following Feeding of Poliovirus Strains

Virus Titer in Serum is Listed as TCD_{50}/0.1 ml.

Guanidine Dosage	Monkey No.	Israel Strain Days after virus challenge 3	5	7	10	Monkey No.	Houston Strain Days after virus challenge 3	5	7	10	Monkey No.	Mahoney Strain Days after virus challenge 3	5	7	10
	749	0	60	16	0	752	16,000	16	0	.	757	16,000	320	0	.
LOW	750	60,000	+	0	0	753	32	1,600	32	0	758	600	1,600	0	0
	751	0	0	0	32	754	0	60	60	0	759	16	6,000	0	0
	756	60,000	600	.	.	755‡	0	0	0	0	760	0	3,200	0	0
	762†	0	0	0	0	765	60	3,200	60	.	768	320	600	600	0
HIGH	763‡	0	16	0	0	766‡	0	0	.	.	779	0	0	0	.
						767	0	0	.	0					
	775	0	0	.	0	773†	160	320	16	0	784	16,000	16,000	16	0
NONE	776	1,600	3,200	0	0	780	0	0	0	0	785	320	6,000	16	0
	777	0	.	0	0	781	600	3,200	0	0	786	0	0	320	320
	778	0	0	0	0	782†	0	16	16	.	787†	0	0	0	0

+ = viremia, titration not done.
0 = no viremia.
† = Animals in which viremia was found in the absence of clinical and histopathologic indication of polio (#773, 782) or clinical and histopathological indication were present but viremia was not found (#762, 787).
‡ = Animals for which it was difficult to establish the cause of death (#775, 763, 766—see text discussion of Table 1).

which was at low titer and occurred only once. The other monkey (#762) in this group, although lacking viremia, showed all the other indications of poliomyelitis, including virus in the CNS. This strain of poliovirus produced viremia in only 1 of the 4 control animals, but all of the low-guanidine group developed viremia.

For the challenge with the Houston strain, only 1 of 3 animals receiving the high guanidine dosage developed viremia, while viremia was detected in all 4 of the low guanidine group, and in 3 of 4 control monkeys. In two of the control animals, #773 and 782, viremia was the sole evidence of poliovirus multiplication.

Of the two animals receiving high guanidine dosage and fed Mahoney strain, only 1 developed viremia, while viremia appeared in all 4 of the low guanidine group, and in 3 of the 4 control animals. The one control animal lacking viremia (#787) gave clinical and histopathological evidence of having developed poliomyelitis.

Table 3 summarizes the results in regard to clinical signs of

TABLE 3

Summary: Poliomyelitis in Cynomolgus Monkeys After Oral Challenge with Type 1 Poliovirus as Related to Treatment with Guanidine

Guanidine Dosage	Number of monkeys positive/number tested Animals challenged with: Israel Strain	Houston Strain	Mahoney Strain	Totals
	Clinical Signs of Poliomyelitis			
Low	4/4	3/3	4/4	11/11
High	1/1	1/2	1/2	3/5
Controls	1/4	1/4	4/4	6/12
	CNS Lesions of Poliomyelitis			
Low	4/4	3/4	4/4	11/12
High	1/2	1/3	1/2	3/7
Controls	1/4	1/4	4/4	6/12
	Poliomyelitis Viremia			
Low	4/4	3/4	4/4	11/12
High	1/2	1/3	1/2	3/7
Controls	1/4	3/4	3/4	7/12
	Antibody Development*			
Low	3/3	1/1	...	4/4
High	...	0/1	0/1	0/2
Controls	3/4	4/4	...	7/8

* Tested in animals surviving 4 weeks or more.

poliomyelitis, histopathology and viremia, respectively. All eleven animals receiving the low dosage of guanidine developed clinical signs of poliomyelitis (top section of Table 3). On the other hand, in the group treated with the high dosage of guanidine, 3 of 5 became ill with poliomyelitis, and in the control group, only 6 of 12.

The three animals which died from causes other than poliomyelitis could not be evaluated clinically but are included in the summary of histopathologic results (middle section, Table 3). As can be seen, 3 of 7 monkeys on the high guanidine dosage developed poliomyelitis lesions in the CNS. Positive histopathologic results also occurred in 11 of 12 receiving low guanidine, and in 6 of 12 in the control group. In all positive monkeys poliomyelitis lesions were present in the lumbar, thoracic, and cervical portions of the spinal cord as well as in the brain stem.

The detection of poliomyelitis viremia (lower middle section, Table 3) followed a similar pattern. For the treated groups, viremia was found in 14 of 19 monkeys, as against 7 positive animals among 12 controls.

Antibodies were tested in monkeys which were sacrificed after 4 weeks of observation. Positive results were obtained in 7 of 8 controls, 4 of 4 in low guanidine dosage, but in neither of the 2 monkeys on high drug dosage.

Among the control animals, the proportion developing clinical signs of poliomyelitis differed among the strains (1 of 4 monkeys challenged with the Israel strain, and 1 of 4 with the Houston strain, as compared to 4 of 4 of the monkeys receiving Mahoney strain). Two additional control monkeys receiving the Houston strain showed viremia and a positive antibody response but without clinical and histopathologic signs of poliomyelitis.

The use of a stomach tube as a possible factor. A stomach tube was used 3 times each day to administer guanidine to the treated groups. The possibility exists that its use three times daily in the guanidine-treated groups might have been a provoking influence to poliomyelitis, which was lacking in the control group. If the tubing process is a factor, then the monkeys receiving high guanidine dosage might be regarded as protected to some degree over those receiving the low guanidine dosage, in which all eleven became ill with poliomyelitis. It is noteworthy that the 2 of 5 animals surviving the high guanidine and poliovirus administra-

tions failed to show viremia, and failed to produce antibodies. They were also free of poliomyelitis lesions in the CNS.

In the report of Rightsel et al. (6), suggestive protective results were obtained in monkeys given near toxic levels of guanidine by mouth and challenge virus in the muscle. If their results and ours have protective significance, it is only to suggest that other compounds should be studied, for the guanidine effect could be demonstrated only at high levels that could not be well tolerated.

Enterovirus excretion. Table 4 presents the detailed data on enterovirus isolation from feces. It should be noted that the virus challenge was not given until the third day after administration of guanidine. In view of the lack of polio antibodies prior to the virus feeding, the viruses isolated in the first 3 collections were presumably nonpolio enteroviruses. From the excretion patterns found, it would appear that the enteroviruses isolated after the poliovirus challenge were similar to those present before virus administration. In only two animals (#753 and 757, in the low guanidine group) was virus excreted before guanidine administration and not subsequently.

In Table 5, the data on virus excretion are summarized. After receiving low dosages of guanidine, 9 of 12 monkeys excreted enteroviruses, whereas 10 of 10 were excreting viruses before guanidine administration began. For the high guanidine group, the 3 monkeys tested before treatment were excreting viruses, and all those tested after treatment began (6 of 6) also excreted viruses. Among the control animals, only 2 of 6 tested were excreting viruses at the beginning of the study period, but all excreted viruses following the time of guanidine administration. The high isolation rates observed are in keeping with an earlier study which we reported (3).

Drug resistant virus. In an attempt to determine whether virus progeny recovered from treated (high dosage) animals had become resistant to the drug, two strains (Mahoney) recovered from the blood of 2 different monkeys were tested for susceptibility to guanidine in tissue culture. In control tests using the parent Mahoney virus, 50 μg of guanidine·HCl inhibited poliovirus so that it grew only 1/100,000 as well as in its absence. However strains recovered from the 2 monkeys receiving guanidine grew 1,000 times more efficiently in the presence of guanidine than did the parent virus (8).

TABLE 4

Excretion of Viruses by Cynomolgus monkeys in relation to the administration of guanidine

Guanidine Dosage	Virus Excretion By monkeys fed Israel strain: Monkey No.	Day before or after guanidine administration began: −9	0*	3	10	17	Virus Excretion By monkeys fed Houston strain: Monkey No.	Day before or after guanidine administration began: −9	0*	3	10	17	Virus Excretion By monkeys fed Mahoney strain: Monkey No.	Day before or after guanidine administration began: −9	0*	3	10	17
	749	+	+	+	+	0	752	+	0	+	..	..	757	+	..	..	0	..
	750	+	..	+	..	+	753	+	..	0	0	..	758	+	0	+	+	..
Low	751	+	+	+	0	+	754	+	..	+	+	+	759	..	..	0	0	..
	756	+	..	+	+	..	755	+	..	+	0	..	760	+	..	+	..	..
	762	..	..	0	+	+	765	+	0	+	0	..	768	..	..	+	+	..
High	763	0	+	..	+	0	766	?	..	?	..	..	779	..	..	+	+	+
							767	+	..	?	?	+						
	775	0	..	0	+	0	773	+	..	?	+	+	784	..	..	+	+	+
Controls	776	+	..	+	0	+	780	..	0	0	+	+	785	..	..	+	+	+
(no	777	..	..	0	0	+	781	..	..	0	0	+	786	..	0	+	+	+
guanidine)	778	?	..	+	0	+	782	..	..	0	+	..	787	..	0	0	+	0

* Day 0 = day guanidine treatment began. (Specimen was collected just prior to first administration). Poliovirus challenge was given on the third day after the first administration of guanidine.

+ = virus isolated from feces.

0 = no virus isolated from feces.

? = uncertain.

TABLE 5

Summary: Excretion of enteroviruses by Cynomolgus monkeys before and after administrations of guanidine

Guanidine dosage	Number of monkeys excreting enteroviruses/number tested							
	Group fed Israel strain		Group fed Houston strain		Group fed Mahoney strain		Totals	
	Pre-guanidine*	Post-guanidine*	Pre-guanidine	Post-guanidine	Pre-guanidine	Post-guanidine	Pre-	Post-
Low	4/4	4/4	3/3	3/4	3/3	2/4	10/10	9/12
High	1/1	2/2	2/2	2/2	...	2/2	3/3	6/6
Controls	1/2	4/4	1/2	4/4	0/2	4/4	2/6	12/12

* Pre = before guanidine administration began.
Post = samples collected after guanidine administration was started and during the period when it was being given.

Conclusions

Guanidine is an efficient inhibitor of poliovirus multiplication in tissue culture (1,6) but the drug did not decrease the incidence or severity of experimental poliomyelitis induced in cynomolgus monkeys by the oral route.

The compound exerted no effect on the excretion of the naturally occurring simian enteroviruses, even in a guanidine dose range that was at toxic levels for the monkey.

After multiplying in monkeys receiving high daily doses of the drug, the virus became resistant to the action of guanidine, similar to the development of antibiotic resistant bacteria. This poses one more problem in the development of a satisfactory viral chemotherapeutic agent.

REFERENCES

1. Crowther, D., and J. L. Melnick: Studies on the inhibitory action of guanidine on poliovirus multiplication in cell cultures. *Virology*, 1961, (In press).
2. Hoffert, W. R., Bates, M. E., and Cheever, F. S.: Studies of enteric viruses of simian origin. *Am. J. Hyg.* 68: 15–30, 1958.
3. Hsiung, G. D., and J. L. Melnick: Orphan viruses of man and animals. *Annals N.Y. Acad of Science* 70: 342, 1958.
4. Melnick, J. L.: Tissue culture methods for the cultivation of poliomyelitis and other viruses. *Diagnostic Procedures for Viral and Rickettsial Diseases, Am. Publ. Hlth. Assoc.* 2nd ed., 97, 1956.
5. Melnick, J. L., and Opton, E. M.: Assay of poliomyelitis neutralizing antibody in disposable plastic panels. *Bull. World Hlth. Org.* 14: 129–146, 1956.
6. Rightsel, W. A., J. R. Dice, R. J. McAlpine, E. A. Timm, I. W. McLean, Jr., G. J. Dixon, F. M. Schabel, Jr.: *The antiviral effect of guanidine.* Science, 1961, in press.
7. Rightsel, W. A., P. Schultz, D. Muething, and I. W. McLean, Jr.: Use of vinyl plastic containers in tissue cultures for virus assays. *J. Immun.* 76: 464, 1956.
8. Melnick, J. L., D. Crowther, and J. Barrera-Oro: Rapid development of drug resistant mutants of poliovirus. *Science*, 1961, (in press).

FASTING AND SMALL INTESTINAL TRANSIT*

IVAN E. DANHOF

Increased intestinal motility as evidenced by increased number and fluidity of stools has been shown to frequently accompany severe limitation of food intake or inanition in the human (6). Semistarvation in rats (7, 16) was accompanied by an increase in small intestinal propulsive motility. While the increase in gastric contractions during periods of hunger or decreased food intake has been attributed to a mild prevailing hypoglycemia (2, 8), the effect of lowered blood glucose levels on small intestinal motility remains obscure. The purpose of the present study was to investigate in the rat factors which might be responsible for increased small intestinal transit incident to prolonged fasting.

Methods

Thirty-six male Sprague-Dawley albino rats, previously maintained on supplemented laboratory rat chow and water *ad libitum*, weighing 170 to 208 grams, were divided into 6 groups of 6 rats each and fasted for 0, 6, 26, 52, 79, and 99 hours, respectively. Water was permitted *ad libitum*. Body weights were recorded daily during the fasting period. After fasting the number of hours indicated, each member of the group (except the unfasted group) was given intragastrically 2 cc. per 100 grams fasted body weight of a suspension of 10% charcoal in 10% gum acacia. Thirty minutes later, the animal was sacrificed by a blow on the head, the abdominal cavity immediately opened, the gastrointestinal tract removed, and the per cent of small intestine traversed estimated using the Macht technic as modified by Van Liere *et al.* (13, 15). Coincident with the removal of the digestive tract, duplicate arterial blood specimens were obtained and the glucemic levels determined by the Hagedorn-Jensen method (4). Samples of liver and the gastrocnemius of the right hind limb were obtained, dissected free of fascia and connective tissue, blotted free of excess blood, placed in tared test tubes containing 30 per cent potassium hydroxide, immersed in a boiling water bath until tissue dissolution commenced, and the glycogen concentration estimated using the method of Goode, Kramer, and Somogyi (3). The determination of glucose obtained from acid hydrolysis of the glycogen was made by the procedure of Shaffer and Somogyi (11). The adrenal glands were dissected free from fat

* From the Department of Physiology, University of Texas Southwestern Medical School, Dallas, Texas. Submitted for publication May 15, 1961.

and connective tissue, sealed in parafilm packets to prevent dehydration, and later weighed on a milligram balance.

Experimental Results

The data are presented in Table 1. With continued fasting a progressive increase in the small intestinal transit was observed. The blood sugar levels showed considerable variation, *i.e.*, increased glucose concentrations during fasting days one and two followed by reduced glucose concentrations to near the control level. A marked reduction in liver glycogen was noted to occur at 26 hours of fasting followed by small but progressive increases with continued inanition. The skeletal muscle showed progressive diminution in glycogen concentration as fasting progressed. The adrenal weights after showing no significant change during the first three days of fasting progressively increased after the 52nd hour of fasting.

Discussion

The progressive increase in small intestinal transit with continued fasting could not be correlated with the prevailing glucose levels as has been reported with respect to gastric motility (2). The reported gradual diminution in blood glucose over the first 2 to 3 days of fasting was not seen to occur (5). The increased glucose levels during this period of fasting may be related to increased utilization of body fat or protein (10). Roberts, Samuels, and Reinecke (9) have presented evidence for the concept that foodstuffs predominantly burned by the extra-hepatic tissues during the early stages of fasting corresponded to the major constituents of the previous diet. The animals in this study prior to the fasting period were maintained on a moderately high protein diet. Since the liver glycogen was significantly depleted by 26 hours of fasting, as has been previously observed (5), the blood glucose level was probably maintained by gluconeogenesis induced by increased adrenal glucocorticoid secretion at the expense of body protein (14). This is corroborated, in part, by the observed losses in body weight.

Skeletal muscle glycogens gradually declined with fasting in a manner similar to reported values (5).

While the adrenal gland weights were not significantly in-

TABLE 1

Small intestinal transit, aspects of carbohydrate metabolism and adrenal weights in fasting male albino rats

Length of fast (hours)	Animals			Small intestinal transit per 30 minutes (%)	Blood glucemic levels (mgms. %)	Tissue glycogens†		Adrenal weight (mgms./100 gms. body wt.)
	Number	Original weight (gms.)	Sacrifice weight (gms.)			Liver (mgms./100 gms. wet wt. of tissue)	Skeletal muscle (mgms./100 gms. wet wt. of tissue)	
0	6	194	194	.....	92.7 ± 2.7	1034 ± 32	458 ± 35	23.4 ± 2.1
6	6	192	190	51.7 ± 3.2‡	88.6 ± 3.8	474 ± 32	462 ± 31	22.5 ± 2.4
26	6	200	189	64.4 ± 2.8	105.3 ± 4.8	83 ± 16	434 ± 14	21.9 ± 2.4
52	6	171	152	74.6 ± 4.7	108.5 ± 2.7	195 ± 27	311 ± 21	23.9 ± 4.4
79	6	173	147	78.3 ± 2.8	88.1 ± 3.3	248 ± 32	216 ± 15	26.9 ± 1.5
99	6	176	136	84.7 ± 6.3	93.1 ± 4.2	352 ± 31	244 ± 16	31.7 ± 2.2

† Expressed as glucose.

‡ S.E. $= \sqrt{\frac{\Sigma\, d^2}{N(N-1)}}$

creased until 79 hours of fasting, the fact that the blood sugar levels were initially maintained above control levels and that the liver glycogen gradually increased with continued fasting suggested that increased glucocorticoid secretion probably commenced upon depletion of liver glycogen stores. Certainly a finite time period following continued application of starvation stress would be required for adrenocortical hypertrophy to be made manifest.

While large doses of adrenocortical extract given to adrenalectomized rats inhibited the *in vivo* transit of intraluminal, dyed carboxymethyl cellulose, moderate doses (as stress-related physiological doses) stimulated small intestinal transit (12). Streeten *et al.* (12) have suggested that aldosterone and other mineralocorticoids were the responsible intestinal stimuli. Only larger doses of glucocorticoids exceeding the levels administered in the adrenocortical extract would influence the intestinal transit in a stimulatory manner similar to smaller doses of aldosterone and other salt-retaining steroids. It remains to be shown conclusively that mineralocorticoids are secreted in increased amounts during prolonged food deprivation; however, significant increases in aldosterone secretion have been shown to occur in rats which were only salt depleted (1). In the present study not only salt, but other alimentation as well was unavailable to the animals. It is assumed in view of the demontrated adrenal hypertrophy that both gluco- and mineralocorticoid secretions were increased.

Summary

Small intestinal transit in the rat progressively increased with fasting up to 99 hours. The increase in intestinal propulsive motility could not be correlated with the blood glucose concentrations. During the first two days of fasting the blood glucose levels were adequately maintained in the face of significant liver glycogen depletion. Thereafter, small progressive increases in liver glycogen were observed while the blood glucose concentrations remained near control levels. Skeletal muscle glycogen concentrations gradually diminished with fasting. Significant adrenal hypertrophy, as evidenced by increased gland weights, occurred after 79 hours of fasting. The possible relation of increased adrenocortical secretion to increased small intestinal motility was discussed.

Acknowledgment

The author wishes to thank Mrs. Elinor Reinmiller, Associate Librarian, University of Texas Southwestern Medical School, for assistance in the literature researches.

REFERENCES

1. Einstein, A. B., and P. M. Hartroft: Alterations in the rat adrenal cortex induced by sodium deficiency: steroid hormone secretion. *Endocrinology* 60: 634–640, 1957.
2. Goldberg, E. M., I. F. Stein, and K. A. Meyer: Studies on gastrointestinal motility. *Gastroenterology* 28: 656–668, 1955.
3. Goode, C. A., H. Kramer, and M. Somogyi: The determination of glycogen. *J. Biol. Chem.* 100: 485–491, 1933.
4. Hagedorn, H. C., and B. N. Jensen: Zur Mikrobestimmung des Blutzuckers mittels Ferricyanid. *Biochem. Z.* 135: 46–58, 1923. Die Ferricyanidmethode zur Blutzucker Bestimmung. II. *Biochem. Z.* 137: 92–95, 1923.
5. Hershey, J. M., and M. D. Orr: The removal of glycogen from living muscle. *Tr. Roy. Soc. Canada*, Sect. V., 22: 151–157, 1928.
6. Keys, A., J. Brozek, A. Henschel, O. Mickelsen and H. L. Taylor: *The Biology of Human Starvation.* Minneapolis: Univ. of Minnesota Press, Vol. I, pp. 538–540; 551–557; 593–596, 1950.
7. Menville, L. J., J. N. Ane and S. N. Blackberg: Observation on the motility of the gastro-intestinal tract of starving rats. *Proc. Soc. Exp. Biol. and Med.* 28: 249–250, 1930.
8. Quigley, J. P., and K. R. Phelps: The mechanism of gastric motor inhibition from ingested carbohydrate. *Am. J. Physiol.* 109: 133–138, 1934.
9. Roberts, S., L. T. Samuels, and R. M. Reinecke: Previous diet and the apparent utilization of fat in the absence of the liver. *Am. J. Physiol.* 140: 639–644, 1944.
10. Samuels, L. T., R. M. Reinecke, and H. A. Ball: Effect of diet on glucose tolerance and liver and muscle glycogen of hypophysectomized and normal rats. *Endocrinology* 31: 42–45, 1942.
11. Shaffer, P. A., and M. Somogyi: Copper-Iodometric reagents for sugar determination. *J. Biol. Chem.* 100: 695–713, 1933.
12. Streeten, D. H. P., B. I. Hirschowitz, K. S. Henley, and H. M. Pollard: Effects of adrenocortical steroids on the propulsive motility of small intestine. *Am. J. Physiol.* 189: 108–112, 1957.
13. Stickney, J. C., E. J. Van Liere, and D. W. Northup: Correlation between propulsive motility and length of the small intestine. *Am. J. Physiol.* 167: 399–402, 1951.
14. Swingle, W. W., and J. W. Remington: The role of the adrenal cortex in physiological processes. *Physiol. Rev.* 24: 89–127, 1944.
15. Van Liere, E. J., W. V. Crabtree, D. W. Northup, and J. C. Stickney: Effect of anoxic anoxia on propulsive motility of the small intestine. *Proc. Soc. Exp. Biol. and Med.* 67: 331–332, 1948.
16. Van Liere, E. J., H. S. Fang, and D. W. Northup: Effect of semistarvation on propulsive motility of the small intestine. *Proc. Soc. Exp. Biol. and Med.* 83: 98, 1953.

SUBMICROSCOPIC MORPHOLOGY OF AVIAN NEOPLASMS V. STUDIES ON NEPHROBLASTOMA[a, b]

L. DMOCHOWSKI,[c] C. E. GREY,[c] B. R. BURMESTER,[d] AND W. G. WALTER[e]

Burmester and his associates (13) have demonstrated that cell-free preparations of erythroblastosis strain R (Beard) induce visceral lymphomatosis in a high percentage of chickens that survived erythroblastosis. They have also shown that chickens inoculated with cell-free preparations of myeloblastosis strain A (Beard) virus, previously known to induce only myeloblastosis (1, 2, 4), developed myeloblastosis, visceral lymphomatosis, renal adenocarcinomas, and osteopetrosis (13). This strain of fowl leukosis obtained from Dr. E. P. Johnson (47) was originally derived by Hall, Bean, and Pollard (42) from a naturally occurring case of neurolymphomatosis. They described the case as the BAI strain A, which caused either myeloblastosis or erythroblastosis or both types of avian leukosis.

The induction of renal epithelial tumors in chickens by cell-free or cell-containing preparations of a leukosis strain (ES-4 or erythroid leukemia-sarcoma strain of Engelbreth-Holm) was previously reported by Carr (14), but attempts to propagate these tumors with cell-free material resulted only in erythroleukemia (14). Recently, Carr (15) demonstrated the cell-free transmission of a renal carcinoma induced by the Mill Hill 2 reticuloendothelioma virus. The induction of kidney tumors with myeloblastosis (Beard) virus has also been reported by Thorell (58).

[a] Supported in part by Grant C-4140 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[b] Presented at the Eighteenth Annual Meeting of the Electron Microscope Society of America, August 29–31, 1960, Milwaukee, Wisconsin. Submitted for publication May 15, 1961.

[c] Section of Virology and Electron Microscopy, The University of Texas M. D. Anderson Hospital and Tumor Institute, and Department of Microbiology, Baylor University College of Medicine, Houston, Texas.

[d] United States Department of Agriculture, Agricultural Research Service, Regional Poultry Research Laboratory, Animal Husbandry Research Division, East Lansing, Michigan.

[e] Present address: United States Department of Agriculture, Agricultural Research Service, Animal Diseases and Parasite Research Division, Plum Island Animal Disease Laboratory, Greenport, Long Island, New York.

These tumors could be transmitted by cell-free preparations (59) and serially transplanted (58).

In a study of the transmission of renal tumors induced by the myeloblastosis (Beard or BAI-A) virus, Walter, Burmester, and Cunningham (61) have observed that these tumors can be propagated by cell transplants. Cell-free preparations from various consecutive renal tumor transplants induced an increased incidence of renal tumors and a decreased incidence of granuloblastosis (myeloblastosis), with the incidence of visceral lymphomatosis and osteopetrosis remaining approximately the same (61). The nephroblastoma, which can be induced in post-embryonic life by a leukemia virus, may have to be included in the leukosis group of avian neoplasms (61).

Studies on submicroscopic morphology of some of the forms of avian leukosis complex have already been reported by us (19–25) and by others (3–8, 11, 45, 46, 53, 54). It appeared, therefore, of interest to study the submicroscopic structure of the original (virus-induced) and transplanted nephroblastomas and to compare the results of such study with those of the studies of submicroscopic appearance of granuloblastosis (strain BAI-A) and of erythroblastosis strain R. Preliminary observations on the presence of characteristic virus particles in the original virus-induced nephroblastoma (kidney adenocarcinoma) and in the transplanted tumor after serial passage have already been reported (26). In the present study an attempt is made to present some similarities and differences between the submicroscopic structure of nephroblastomas, both virus-induced and transplanted, and that of granuloblastosis (myeloblastosis BAI-A strain) and of erythroblastosis strain R.

Materials and Methods

The nephroblastomas examined in the present study were obtained from chickens of an inbred line of single-comb White Leghorns selected at the Regional Poultry Research Laboratory (line 151) for susceptibility to lymphomatosis and maintained in isolation for 17 years to reduce the extent of natural infection (62, 63). The tumors developed in chickens inoculated by the intravenous route, when 14 days old, with –1.5 and –2.5 log dose of myeloblastosis virus in plasma from chickens with myeloblastosis virus obtained from Dr. J. W. Beard (13).

In addition to the virus-induced nephroblastomas, tumors following serial transplantation were studied. The transplanted tumors were obtained from

chickens used in cell transplantation experiments started with a pool of two nephroblastomas which developed in chickens inoculated with the myeloblastosis virus (strain BAI-A). Original donors developed the tumors at 160 days after inoculation.

The tumor cell suspension was made by mincing freshly harvested tumor tissue in an Olson homogenizer (52) and diluting the resulting homogenate in two parts of Simm's salt solution (55). The cell suspension was then filtered through sterile gauze, and following addition of antibiotics (1250 units of penicillin G and 2.5 mg of dihydrostreptomycin per ml) used for inoculation in 0.2 ml quantities into chickens (61). First passage with cell suspension was carried out by the intraperitoneal route, and all the subsequent passages (2–13) in the pectoral muscle. In general, tumors became palpable in 10–14 days and were harvested at 3–4 weeks for further passage (61). Tumors from the 13th transplant were used for the present study. Cell-free extracts were prepared by Walter, Burmester and Cunningham (61) from tumors of passages 2, 8 and 12a and inoculated intravenously in 0.2 ml quantities into 7-day-old White Leghorn chickens of line 151. The results obtained with these cell-free extracts will be reported elsewhere (61) and briefly discussed in the present paper in connection with the results of electron microscope studies of the transplanted tumors.

The tissues for study in the electron microscope were prepared by procedures based on fixation in veronal-acetate buffered osmium tetroxide and methacrylate embedding similar to that described for the study of erythroblastosis strain R (23), granuloblastosis(25), visceral lymphomatosis (24), and erythroblastosis of RPL-12 strain (21). In order to obtain better preservation of specimens, ultrathin sections cut with glass knives on a Porter-Blum microtome, were dipped in suitably prepolymerized butyl methacrylate (a procedure developed by C. E. Grey). The sections, stained with uranyl nitrate (64), were examined in an RCA EMU 3E electron microscope.

Results

General.

A histological study of the renal tumors, both the original and transplanted, revealed that the tumors are embryonal nephromas, histologically comparable with Wilm's tumor of humans (61). Considerable variation in the degree and amount of differentiation of stroma and epithelium was found in this tumor with areas of cartilage and bone (61). The filtrate-induced tumors were of similar histological appearance to that of tumor transplants, and showed a predominance of either epithelial or sarcomatous cells (61). Individual tumors or areas resembled adenocarcinomas or adenosarcomas (61). The term embryonal nephroma suggested by Feldman (34) for this type of tumor in domes-

tic animals was replaced by the term nephroblastoma (33) and has been applied to renal tumors induced by granuloblastosis (myeloblastosis) strain BAI-A virus (61).

The electron microscope studies were greatly facilitated by the above-mentioned histological studies. As far as general appearance of cells and their constituents is concerned, no difference was observed between the cells of virus-induced (original) and of the transplanted tumors. No changes, which could be described as characteristic, were found in the nuclei, nucleoli or the endoplasmic reticulum. The shape of the nuclei was variable, and the nucleoplasm was densely granular and filamentous (Figs. 1, 7, 9, 12), the nucleus occasionally showing invaginations (Fig. 1). Chromatin in variable amounts centrally or peripherally located, with nucleoli of variable size and location was frequently observed. In the cytoplasm numerous ribonucleoprotein particles were present, both free (Fig. 11) and attached to the endoplasmic reticulum (Fig. 1). Both smooth and rough endoplasmic reticulum was observed (Figs. 1, 9), with the Golgi apparatus (not illustrated) being variable in size and complexity. Occasionally the endoplasmic reticulum was found swollen and filled with homogeneous material of lesser density than that of the surrounding cytoplasm (Fig. 1). Mitochondria of variable size, shape, and number were frequently encountered. They showed considerable variation in their internal structure, from fully preserved with distinct cristae to complete loss of internal structure with vacuolization.

Cytoplasmic Virus Particles.

Two sites of cytoplasmic virus particles have been found in the tumor cells. One site is in inclusion bodies, presumably of mitochondrial origin (Figs. 7, 8). A second site occurs within aggregates of the cytoplasm (Figs. 7, 9, 9a, 10, 11).

The outer membranes of the cytoplasmic inclusion bodies are often more dense than those of normal appearing mitochondria, and their matrix is usually densely osmiophilic showing structures which appear to be remnants of membranes (Figs. 7, 8). In some of the larger bodies with a dense matrix, no internal or outer limiting membranes can be found. In what may be the last stage of formation of the inclusion bodies, it is difficult to determine whether the virus particles have been phagocytized or whether

they are being released as a result of cells breaking down. Within the inclusions fully formed virus particles with a nucleoid and surrounding dense membrane may frequently be seen (Figs. 7, 8).

The virus particles within inclusions of possible mitochondrial origin in both the original virus-induced and transplanted nephroblastomas show a variation in size from 625Å to 870Å, with the greatest number measuring approximately 760Å. The size range of the particles is more limited than that encountered in other intra- and extra-cytoplasmic virus particles.

The second site of cytoplasmic virus particles can be found in the cytoplasmic aggregates, which are composed of small dense osmiophilic particles of the size and appearance of ribonucleoprotein granules (Figs. 7, 9, 9a, 10, 11). These aggregates vary in size and have been usually found in the outer part of the cytoplasm (Figs. 7, 9). The granules are frequently seen to aggregate in size similar to that of the nucleoid of fully formed virus particles (Figs. 9a, 10). Within the aggregates, described as viral matrix, various structures may be observed which could be interpreted as virus particles in different stages of formation (Figs. 9a, 10). Fully formed virus particles gradually replace the viral matrix (Fig. 11) and are released into the intercellular spaces or the lumen of tubules (Fig. 12) following either the breakdown of the cell or vacuolization of the affected part of the cytoplasm. The gradual emergence of virus particles from viral matrix of the cytoplasm is illustrated in Figures 9a, 10, and 13. The virus particles may also be observed within viral matrix released or extruded by the cells and present in the lumen of the tubule (Figs. 12, 13).

In many cells the viral matrix is present in the cytoplasm showing inclusion bodies of probable mitochondrial origin (Fig. 7). The virus particles which are found in the viral matrix frequently show an eccentrically located nucleoid, and have a wider size range than that encountered in the cytoplasmic inclusions, varying from 600Å to 1250Å in diameter (Figs. 11, 11a). The particles most frequently encountered are of 800Å, 875Å and 1000Å diameter.

No transitional stages could be found between the two sites of cytoplasmic virus particles. An additional site has frequently

been encountered, usually occurring in cells showing one or both of the other two sites. The evidence so far obtained indicates that both the cortical cytoplasm and plasma membrane appear to participate in the formation of virus particles at the cell border (Figs. 1, 4, 5, 6, 7, 11). The various stages in possible particle formation by the cell membrane may be seen in Figures 5, 6 (a–h). These may be described in the following sequence: thickening of the cellular membrane which separates into two well-defined membranes, gradually increasing protrusion or "budding" with narrowing and pinching off of the particle. The particles appear either as structures with two concentric rings or as structures with a dense center gradually increasing in size. Some virus particles appear to be attached to the cellular membrane by a filament of varying thickness, and some show only a double membrane without a dense nucleoid (Figs. 4, 5, 6). The relationship of the two types of particles, those with and without the nucleoid, remains uncertain. The particles with two membranes without the dense center may be a stage in the formation of the complete virus particles containing the nucleoid.

Intercellular Virus Particles.

Large numbers of intercellular virus particles have been observed in many sections both of the original virus-induced and of the transplanted nephroblastomas. No relationship could be established between the number of cytoplasmic particles and that of intercellular virus particles. Many fully developed intercellular virus particles have been observed near the "budding" processes of cells (Figs. 1, 7, 11). They have also been found in the lumen of tubules of the nephroblastomas (Figs. 2, 3). The intercellular virus particles in the original virus-induced nephroblastomas are largest, ranging in size from 500Å to 1400Å in diameter. Virus particles of approximately 1000Å were most frequently encountered. The particles observed in the intercellular spaces and tubules of the transplanted nephroblastomas are somewhat smaller in size, ranging from 600Å to 950Å, with the most frequently encountered size of 800Å.

Granuloblastosis (Myeloblastosis BAI-A Strain).

In view of the changes observed in the cells of nephroblastoma

induced by the myeloblastosis strain BAI-A virus, it appeared of interest to re-examine organs and tissues from chickens with granuloblastosis induced by this virus. Spleen and bone marrow of the diseased chickens were studied. In the spleen, virus particle formation appears to take place within cytoplasmic inclusions, which could be interpreted as being of mitochondrial origin (Figs. 14, 14a, 16). In spite of intensive search, no other sites of intracellular virus particles formation could be found. However, in the cells of myeloid origin present in the bone marrow of the diseased chickens, in addition to the presence of virus particles within cytoplasmic inclusions the cellular membranes appear to participate in the formation of virus particles (Figs. 15c, 15d). The type of cells in the spleen in which the intercellular particles have been observed may be seen in Fig. 14. These cells are granuloblasts and macrophages. Virus particles are present in the intercellular spaces in both spleen and bone marrow. The size of virus particles, observed in the cytoplasmic inclusions of probable mitochondrial origin, has been found to vary from 500Å to 780Å, with the largest number of particles in the 670Å range. The size of particles in the intercellular spaces is somewhat larger, with particles of 800Å in diameter predominating in number.

Erythroblastosis Strain R.

In view of the apparent difference in the sites in which cytoplasmic virus particles appear to be formed in nephroblastomas and granuloblastosis (myeloblastosis BAI-A strain), it appeared of interest to re-examine organs from chickens with erythroblastosis strain R (Beard). In the spleen of the diseased chickens, virus particles have been observed in the intercellular spaces, in cytoplasmic inclusions of probable mitochondrial origin, and in intracytoplasmic vacuoles, some of which could be interpreted as due to degeneration of mitochondria (Figs. 17, 17a, 18). The cytoplasmic particles have been observed in the cells of erythroid origin, in macrophages and histiocytes. In spite of prolonged search, no cytoplasmic aggregates, similar to those found in nephroblastomas, could be observed in the spleen or bone marrow of chickens with erythroblastosis or myeloblastosis. Plasma membranes of cells of erythroid origin also appear to participate in the formation of virus particles (Figs. 15a, 15b). Virus particles in the cytoplasmic inclusions have been found to vary from 550Å

to 820Å, with the largest number of virus particles being of 670Å diameter. Again, as in granuloblastosis, the size of particles in the intercellular spaces has been found somewhat larger, with particles of 800Å in diameter being the most frequently encountered.

Discussion

Successful transmission of nephroblastomas by means of cell-free extracts of the transplanted tumors has been reported by Walter, Burmester, and Cunningham (61). The cell-free preparations from tumors of the 2nd transplant induced granuloblastosis (65%); nephroblastoma (30%); visceral lymphomatosis (12%); and osteopetrosis (17%). Similar preparations from tumors of the 8th transplant induced a much lower incidence of granuloblastosis (5%); a high incidence of nephroblastomas (62.5%); an increased incidence of visceral lymphomatosis (62.5%); and osteopetrosis (17.7%). Cell-free preparations from tumors of the 12a transplant failed to induce granuloblastosis, but caused a high incidence of nephroblastomas (64.9%); they also induced visceral lymphomatosis (18.9%) and osteopetrosis (18.9%). A number of chickens, inoculated with cell-free material from the transplanted tumors, developed two neoplastic conditions in all possible combinations, except for the leukemia and visceral lymphomatosis combination, and some developed three neoplastic conditions (61).

The change in tumor-inducing property of the BAI-A strain of myeloblastosis virus after serial passages of nephroblastoma could be explained in several ways. If the original myeloblastosis virus preparation contained several viruses, segregation and elimination of the virus responsible for myeloblastosis may have taken place (61). It could also be explained on the basis of a single agent with a multiple tumor-inducing property which has changed following repeated tumor passages (61). The possibility of modification of one virus may also have to be considered (61). Many investigators have held the view that avian leukosis strains contain a single virus with a property to induce a distinct range of tumors under specific circumstances (47, 48, 51, 57, 58, 60, 66). Evidence has been presented, in the case of the polyoma virus, that one virus is responsible for the many types of tumors in animals of several species (56). If one virus is responsible for

the neoplasms induced by the BAI-A strain of myeloblastosis, the resistance of the host or loss of titre during serial passages are not likely to be responsible for the lack of leukemic response, in view of the data available from bioassays of the transplanted tumors (61).

In this connection, the data obtained by Walter, Burmester, and Cunningham (61) from transplantation of nephroblastomas appear to be of interest. The tumors were easily propagated in young chickens by cell suspensions, which induced progressive growth at the site of inoculation and led to the death of the host within the comparatively short time of 5 weeks. Some chickens developed also distant tumors, such as nephroblastomas, visceral lymphomatosis, osteopetrosis, and granuloblastosis. This could only be attributed to a virus or viruses released from the transplanted tumor cells (61). In chickens with tumors of the 10th to 12th transplant of nephroblastoma, all other tumors except granuloblastosis appeared (61). These results correspond with those obtained by cell-free preparations from nephroblastomas of the later passages. The age of chickens, used for transplants, cannot be taken as the reason for the disappearance of granuloblastosis, since chickens of similar age (15 days) inoculated with leukemic plasma of lower concentrations than the tumor suspensions developed a higher than 90% incidence of granuloblastosis (61).

Successful transmission of chicken kidney tumors has also been reported by Thorell (58). These tumors were induced by virus of the same origin as that used by Walter *et al.* (61), and by Burmester *et al.* (13). However, the tumors were described as adenosarcomas of the kidneys. Chickens, inoculated with the BAI-A strain virus at the age of 3 days, developed a 100% incidence of granuloblastosis. Similar chickens, inoculated with the same virus preparation, when 14 days old, developed a 50% incidence of leukemia and a 30% incidence of kidney tumors. In chickens inoculated with cell-free preparations of the transplanted kidney tumors, the incidence of granuloblastosis decreased to 20%, while the incidence of kidney tumors increased to 80% (59). Thorell (58) considered the possibility of a modified host response in chickens partly resistant to granuloblastosis (myeloblastosis) or the possibility of the presence of several types of leukemia virus in the plasma of leukemic birds as reasons

for the induction of the kidney tumors. He considered the latter possibility as a more likely one, in view of the appearance of kidney tumors, following inoculation of 3-day-old chickens with leukemic plasma in higher dilutions, and the induction of leukemia only in chickens inoculated with lower dilutions, and the induction of leukemia only in chickens inoculated with lower dilutions of the same plasma. Thorell, however, pointed out that host reactions could not be eliminated in this manner (58). Myeloblastosis (35) and erythroblastosis (36) are the two leukemic forms of the chicken leukosis complex which are considered to be induced by an agent, or agents, distinct but immunologically related (1). According to Thorell (58), the related but not identical myeloblastosis and erythroblastosis viruses of chicken leukemia affect different stem cells in the hematopoietic organ, the leukoblasts and pro-erythroblasts respectively.

Many of the physical, chemical, and biological properties of the myeloblastosis (granuloblastosis) virus have been described (28, 29). Extensive electron microscope studies of sections of organs of chickens with granuloblastosis have been reported by us (18, 19, 20, 22, 25) and by others (6). Characteristic virus particles varying in size from 670Å to 800Å have been observed within the cells and in the intercellular spaces in sections of organs of the diseased chickens. Similar particles have also been observed in sections of centrifugal pellets from plasma of chickens with myeloblastosis (8). A study of myeloblasts from circulating blood of the diseased chickens has shown little evidence of the presence of a virus (54). Examination of myeloblasts grown in tissue culture has revealed mitochondria and osmiophilic bodies, varying in size and shape ("gray bodies"); some of these bodies have been found in the process of vacuolization enclosed by a membrane (11). In some of the bodies, with outlines of mitochondrial cristae, virus particles have been found (54), similar to particles in the centrifugal pellets from plasma of chickens with myeloblastosis (8). The presence of the "gray bodies" in myeloblasts grown *in vitro* has repeatedly been observed (11, 12, 53). Correlative cytochemical and electron microscope studies of myelobasts grown in tissue culture have provided information on the constitution and activity of these bodies (12, 38, 65). There is impressive evidence that the virus particles encountered in reticular cells of the spleen and bone marrow (6) and in the

granuloblasts of the spleen (18, 19, 20, 22, 25) represent the agent responsible for myeloblastosis. From the studies of organs of the diseased chickens and of myeloblasts grown *in vitro*, it appears that virus particles are formed in cytoplasmic inclusions, described as "viroplasts" in some studies (38), which could be interpreted as being of mitochondrial origin. In none of the studies on myeloblastosis, so far described, has the participation of the plasma cell membrane in the formation of virus particles been noted.

Electron microscope studies of organs from chickens with erythroblastosis strain R have revealed, as in myeloblastosis, the presence of extracellular virus particles and particles (670Å) within cytoplasmic inclusions of probable mitochondrial origin and within cytoplasmic vacuoles (18, 19, 20, 22, 23). The virus particles have been observed in "tumorous" cells. This term was used to describe primitive cells of erythroid origin (23) and not the reticular cells or macrophages, which do contain virus particles, but their presence in such cells was considered incidental to the process of malignancy. It may, however, indicate that incomplete and fully developed virus particles are taken up by macrophages and synthesized or replicated, as suggested by Iwakata (45) and Iwakata and Amano (46).

The presence of virus particles (800Å) in the affected organs (spleen and bone marrow) of chickens with erythroblastosis has also been reported by other sources (5, 7, 45, 46). Particles similar to those described in the sections of cells have also been found in sections of ultracentrifugal pellets from infective plasma of chickens with erythroblastosis (8). There is, therefore, strongly suggestive evidence available that the virus particles are the causative agent of erythroblastosis. While in our studies (23) virus particles have been observed chiefly in "tumorous" cells, in other studies (5) they have been found in reticulocytes, macrophages, and paraerythroblasts, in that order of frequency. The difference in the frequency of distribution of virus particles within cells of the different organs may be due to the stage of the disease at which organs of the affected chickens were taken for electron microscope study. It is possible that the presence of virus particles increases in erythroblasts during the last stages of the disease. In all studies mentioned, virus particles have been found in the intercellular spaces, intracytoplasmic vacuoles, and in the

cytoplasmic inclusions. In one study the apparent formation of virus particles by cell membrane of erythroblasts of the bone marrow has been noted (5).

Electron microscope studies of erythroblasts in the bone marrow of the diseased chickens, in the circulating blood, and of erythroblasts grown in tissue culture have recently been reported (44). These studies confirmed essentially the results already discussed and obtained in the affected organs of chickens with erythroblastosis (5, 23, 45, 46). Little evidence of alterations in the structure of erythroblasts from the circulating blood of chickens with erythroblastosis has been observed, but extensive changes have been noted in the erythroblasts grown in tissue (44). Large numbers of virus particles have been found in these erythroblasts, mostly in a matrix of amorphous material outside and around the cells. Virus particles have also been observed "engulfed" in intracytoplasmic vacuoles (44). These vacuoles, however, appear to be enclosed by a single membrane similar in appearance to that of some of the altered mitochondria. Apparent formation of virus particles has been found to take place within certain loci of the cytoplasm of erythroblasts grown *in vitro* (44). In addition, the cellular membrane has been to participate through the "budding" process in the formation of virus particles in erythroblasts maintained in tissue culture (44).

The present findings essentially confirm those already published on myeloblastosis and erythroblastosis by us (18, 19, 20, 23, 25) and by others (5, 6, 7, 45, 46). It appears that formation of virus particles may take place within cytoplasmic inclusions, which could be interpreted to be altered mitochondria of the various types of cells of the spleen and bone marrow of chickens with myeloblastosis (Figs. 14a, 16) and the erythroblastosis (Fig. 18). In some cases, it is indeed difficult to decide whether virus particles are present in the cytoplasmic vacuoles or in the cytoplasmic inclusions which have undergone vacuolization (Fig. 14a), but in other cases, the cytoplasmic inclusions, at least in our opinion (17), appear to be an integral part of the cytoplasm (Figs. 14a, 16, 18).

It is of interest that the apparent formation of virus particles by cell membranes appears to take place both in erythroblastosis (Figs. 15a, 15b) and in myeloblastosis (15c, 15d), although in both diseases, especially in the latter, it has been much less fre-

quently encountered than the virus particles within the cytoplasmic inclusions.

An extensive search failed to reveal any cytoplasmic aggregates within the cells of the spleen and bone marrow of chickens with granuloblastosis and erythroblastosis. This, of course, need not indicate that this site of virus particles is entirely absent in the cells of the affected organs of chickens with myelo- and erythroblastosis. It may, however, point out that virus particles within cytoplasmic aggregates occur much less frequently than virus particles within cytoplasmic inclusions and within cellular membranes.

In nephroblastoma, both virus-induced and transplanted, as in all forms of chicken leukosis which have been studied, virus particles occur within cytoplasmic inclusions, which could be interpreted as being of mitochondrial origin (Figs. 7, 8).

The apparent formation of virus particles by cytoplasmic membranes also occurs in nephroblastoma, both original and transplanted (Figs. 5, 6). It has been much more frequently encountered in nephroblastomas than in granuloblastosis and erythroblastosis. Formation of virus particles at the cell membrane has previously been reported in mouse mammary carcinoma (19, 16, 49) and in certain types of mouse leukemia (43).

A characteristic feature of nephroblastomas, not encountered as yet in any of the chicken leukosis forms, is the presence and what appears to be formation of virus particles within cytoplasmic aggregates described as viral matrix (Figs. 7, 9, 9a, 10, 11). The virus particles are still present within the cytoplasmic aggregates, following the breakdown of cells (Figs. 12, 13), although virus particles cannot continue to be formed in such aggregates outside the living cells. At this stage of investigations, it may be premature to arrive at a conclusion that this form of particle development is characteristic of nephroblastoma and not of any other form of chicken leukosis or of other chicken tumors. Indeed, recent information on Rous sarcoma appears to confirm this doubt.

Extensive studies on the ultrastructure of Rous sarcoma virus and of tumors induced by this agent were carried out by a number of workers (10, 30, 31, 32, 37, 39, 40). The presence and location of virus particles outside the cells and within cytoplasmic vacuoles

of tumor cells were described and the structure of virus particles demonstrated. However, no structures were observed which could be interpreted as different stages of virus particle development in the cytoplasm of tumor cells. An electron microscope study of chick embryo fibroblasts infected with Rous sarcoma, recently carried out (41), has revealed some stages of the development of virus particles, although apparently not a complete cycle, within loci of the cytoplasm, resembling to a certain extent the viral matrix observed in the cytoplasm of nephroblastomas. Particles present in these loci differ somewhat from other Rous virus particles. They have a considerably more developed nucleoid (330Å) but less dense, surrounded by a single fine membrane, which in turn is surrounded by ribonucleoprotein particles. These virus particles measure 440Å and are therefore smaller than the particles present outside the cells. The relationship of the smaller intracytoplasmic particles to the other larger particles is unknown. In addition, characteristic virus particles have been found extracellularly and in cytoplasmic vacuoles, interpreted as invaginations of the cell membranes (41). Similar particles have also been found within certain loci in the cytoplasm of Rous sarcoma cells (41), which correspond to previously described "gray bodies" (11) or "viroplasts" (38) in myeloblastosis.

In the nephroblastomas examined in the present study, some cells have been observed showing all three sites of apparent virus particle development. Cytoplasmic inclusion bodies of probable mitochondrial origin, the cytoplasmic aggregates or viral matrix, and the "budding" process of cellular membranes have been found in that order of frequency. In the material examined, no great difference could be observed between the original and the transplanted nephroblastomas in the frequency of occurrence of the three types of cellular alterations.

In view of the origin of nephroblastomas which were induced by the myeloblastosis BAI-A strain virus and the results of bioassays of cell-free preparations from nephroblastomas after several sequential passages, an attempt was made to analyze the size distribution of virus particles observed in the primary and the transplanted tumors, in an attempt to correlate virus particles of a certain size with the results of bioassays. The size of virus particles observed in the cytoplasmic inclusion bodies in the primary

(virus-induced) nephroblastomas varies from 625Å to 850Å. Particles of 750Å diameter (100 particles counted) were most frequently observed. In the transplanted nephroblastomas, a similar size range of particles was observed, with the most frequently encountered particles (100 particles counted) measuring approximately 800Å. There appears to be no significant difference in the size of virus particles present in the cytoplasmic inclusions in both types of neophroblastomas. The virus particles present within the so-called viral matrix of primary tumors range from 600Å to 1250Å. Particles most frequently found measure 800Å, 875Å, and 1000Å. Particles of the three sizes were encountered in equal numbers (100 particles counted). Virus particles in the viral matrix of the transplanted (13th passage) nephroblastoma range in size from 625Å to 1125Å. Particles of 750Å diameter were observed most frequently (100 particles counted). At this stage of investigation it is difficult to make any comments about the apparent difference in size of the particles present in the viral matrix of the primary and transplanted tumors. The extracellular particles in the primary nephroblastoma vary in size from 500Å to 1350Å, with the largest number of particles measuring 1000Å. The extracellular particles in the transplanted nephroblastoma range in size from 600Å to 850Å, with the largest number of particles being of 800Å diameter (100 particles counted). No difference could be observed between the morphological appearance of the virus particles present in the primary and transplanted nephroblastomas. At present it is not possible to account for the great variation in size of the virus particles in nephroblastomas. They may represent different viruses, or the same virus in different stages of development.

Measurement of virus particles in granuloblastosis, erythroblastosis, and visceral lymphomatosis were previously reported (23, 24, 25). The present study confirms the previous reports. Virus particles present in both granuloblastosis and erythroblastosis vary in size from 550Å to 830Å, with the largest number of particles measuring 670Å (100 particles counted). The size of these particles falls within a narrow zone of the size range of particles present in primary and transplanted nephroblastomas. A comparison of the size of virus particles most frequently encountered in myeloblastosis with that of the virus particles most numerous in nephroblastomas appears to indicate a shift toward a larger size particle in nephroblastomas.

At present there is no evidence that the virus particles of variable size which appear to be formed in several different sites of the nephroblastomas cells are related to each other or even to the tumor itself. No correlation could be established between the particles of different size present in the primary and the transplanted nephroblastomas and the results of bioassays, which demonstrated the gradual increase in the incidence of nephroblastomas and the disappearance of myeloblastosis in chickens inoculated with cell-free extracts of these tumors obtained from sequential passages (61).

Summary

An electron microscopy study of nephroblastomas in chickens (previously described as renal adenocarcinomas) was carried out. Tumors induced by cell-free preparations of the myeloblastosis strain A (Beard), and after serial transplantations in chickens were studied. Cytoplasmic and intercellular virus particles were observed in the induced and transplanted nephroblastomas. Cytoplasmic particles present within cytoplasmic inclusions, probably of mitochondrial origin, and in cytoplasmic vacuoles average 750Å, ranging in size from 625Å to 850Å. Similar particles, somewhat larger in size, are present at the cell border and the cell membranes appear to be involved in their formation. The particles in the intercellular spaces, although similar in appearance, are larger and average 1000Å. In addition, the cytoplasm itself appears to participate in the formation of virus particles. The particles which appear to form within the cytoplasmic aggregates vary in size from 600Å to 1250Å; the most frequently encountered particles measure 800Å, 880Å, and 1000Å. The virus particles are composed of a nucleoid, surrounded by a single or occasionally by a double membrane, while some, probably immature particles contain only double membranes. The relationship of the virus particles to nephroblastoma and to the virus particles observed in myeloblastosis strain A and in erythroblastosis strain R is discussed.

Acknowledgment

Grateful acknowledgment is due to Mr. Frank Padgett for excellent technical assistance.

REFERENCES

1. Beard, J. W.: Etiology of avian leukosis. *Ann. New York Acad. Sc.*, 68: 473–486, 1957.
2. Beard, J. W.: Etiologic individualities of the avian leukemias, myeloblastosis and erythroblastosis. *Proc. of Int. Soc. of Hematology*, 103: 19–33, 1957.
3. Beaudreau, G. S., C. Becker, R. A. Bonard, A. M. Wallbank, D. Beard, and J. W. Beard: Virus of avian myeloblastosis. XIV. Neoplastic response of normal chicken bone marrow treated with the virus in tissue culture. *J. Nat. Cancer Inst.*, 24: 395–415, 1960.
4. Beaudreau, G. S., C. Becker, D. G. Sharp, J. C. Painter, and J. W. Beard: Virus of avian myeloblastosis. XI. Release of the virus by myeloblasts in tissue culture. *J. Nat. Cancer Inst.*, 20: 351–380, 1958.
5. Benedetti, E. L., and W. Bernhard: Recherches ultrastructurales sur le virus de la leucémie erythroblastique du poulet. *J. Ultrastructure Res.*, 1: 309–336, 1958.
6. Benedetti, E. L., W. Bernhard, and C. Oberling: Présence de corpuscules d'aspect virusal dans des cellules spléniques et medullaires de poussins leucémiques et normaux. *Compt. rend. Acad. Sc.*, 242: 3891–3894, 1956.
7. Benedetti, E. L., and R. Leplus: Cytologie de l'erythroblastose aviaire (étude au microscope électronique). *Revue d'Hematologie*, 13: 199–230, 1958.
8. Bernhard, W., R. A. Bonar, D. Beard, and J. W. Beard: Ultrastructure of viruses of myeloblastosis and erythroblastosis isolated from plasma of leukemic chickens. *Proc. Soc. Exper. Biol. & Med.*, 99: 48–52, 1958.
9. Bernhard, W., and M. Guérin: Evaluation quantitative du virus dans des tumeurs mammaires spontanées ou graffées de différentes souches de souris et étude de ces rapports avec l'appareil de Golgi. 2nd Int. *Symp. ou Mammary Cancer, Perugia*, 627–640, 1957.
10. Bernhard, W., C. Oberling, and P. Vigier: L'ultrastructure du particules de virus du sarcome de Rous et leur rapport avec le cytoplasme de cellules tumorales. *Bull. Cancer*, 43: 407–422, 1956.
11. Bonar, R. A., D. F. Parsons, G. S. Beaudreau, C. Becker, and J. W. Beard: Ultrastructure of avian myeloblasts in tissue culture. *J. Nat. Cancer Inst.*, 23: 199–226, 1959.
12. Bonar, R. A., D. Weinstein, J. R. Sommer, D. Beard, and J. W. Beard: Virus of avian myeloblastosis. XVII. *Morphology of progressive virus-myeloblast interactions* in vitro. N. C. I. Monograph No. 4: 251–290, 1960.
13. Burmester, B. R., W. G. Walter, M. A. Gross, and K. Fontes: The oncogenic spectrum of two "pure" strains of avian leukosos. *J. Nat. Cancer Inst.*, 23: 277–291, 1959.
14. Carr, J. G.: Renal adenocarcinoma induced by fowl leukemia virus. *Brit. J. Cancer*, 10: 379–383, 1956.
15. Carr, J. G.: Kidney carcinomas of the fowl induced by the MH2 reticuloendothelioma virus. *Brit. J. Cancer*, 14: 77–82, 1960.
16. Dmochowski, L.: Progress in mammalian genetics and cancer. *J. Nat. Cancer Inst.*, 15: 785–787, 1954.
17. Dmochowski, L.: Viruses and tumors in the light of electron microscope studies: A review. *Cancer Research*, 20: 977–1015, 1960.
18. Dmochowski, L., and C. E. Grey: Studies on submicroscopic structure of leukemias of known or suspected viral origin: A review. *Blood*, 13: 1017–1042, 1958.

19. Dmochowski, L., C. E. Grey, and B. R. Burmester: Observations on the possible life-cycle of certain viruses as revealed in ultrathin sections of chicken leukosis tumors. Proc. 16th Ann. Meeting Electron Microscope Soc. of Am. *Abstract*, p. 5, 1958.

20. Dmochowski, L., C. E. Grey, and B. R. Burmester: Studies on submicroscopic structure of chicken leukosis tumors: lymphomatosis, erythroblastosis, and granuloblastosis. Proc. 7th Int. Cancer Congress, London. *Abstract*, pp. 247–248, 1958.

21. Dmochowski, L., C. E. Grey, and B. R. Burmester: Submicroscopic morphology of avian neoplasms. IV. Studies on erythroblastosis of Strain RPL-12. *Proc. Soc. Exper. Biol. & Med.*, 100: 517–519, 1959.

22. Dmochowski, L., C. E. Grey, and B. R. Burmester: Studies on the submicroscopic structure of chicken leukosis: lymphomatosis, erythroblastosis and granuloblastosis. *Acta Unio Internat. contra cancrum*, 15: 780–790, 1959.

23. Dmochowski, L., C. E. Grey, B. R. Burmester, and A. K. Fontes: Submicroscopic morphology of avian neoplasms. I. Studies on erythroblastosis. *Proc. Soc. Exper. Biol. & Med.*, 98: 662–665, 1958.

24. Dmochowski, L., C. E. Grey, B. R. Burmester, and M. A. Gross: Submicroscopic morphology of avian neoplasms. III. Studies on visceral lymphomatosis. *Proc. Soc. Exper. Biol. & Med.*, 100: 514–516, 1959.

25. Dmochowski, L., C. E. Grey, B. R. Burmester, and W. G. Walter: Submicroscopic morphology of avian neoplasms. II. Studies on granuloblastosis (myeloblastosis). *Proc. Soc. Exper. Biol. & Med.*, 98: 666–669, 1958.

26. Dmochowski, L., C. E. Grey, S. E. Stewart, B. E. Eddy, B. E. Burmester, and W. G. Walter: Morphology of cell constituents in tumors of viral origin. In: *Cell Physiology of Neoplasia.* The Univ. of Texas Press, Austin, Texas, 1960, pp. 185–218.

27. Eckert, E. A., D. Beard, and J. W. Beard: Virus of avian erythroblastosis. I. Titration of infectivity. *J. Nat. Cancer Inst.*, 16: 1099–1120, 1956.

28. Eckert, E. A., D. G. Sharp, I. Green, and J. W. Beard: Virus of avian erythromyeloblastic leukosis. IX. Antigenic constitution and immunological characterization. *J. Nat. Cancer Inst.*, 16: 593–643, 1955.

29. Eckert, E. A., D. G. Sharp, E. B. Mommaerts, R. H. Reeves, D. Beard, and J. W. Beard: Virus of avian erythromyeloblastic leukosis. III. Interrelations of plasma particles, infectivity, and the enzyme dephosphorylating adenosine-triphosphate. *J. Nat. Cancer Inst.*, 14: 1039–1053, 1954.

30. Epstein, M. A.: The identification of the Rous virus; a morphological and biological study. *Brit. J. Cancer*, 10: 33–48, 1956.

31. Epstein, M. A.: Observations on the Rous sarcoma virus; fine structure and relation to cytoplasmic vacuoles. *Brit. J. Cancer*, 11: 268–273, 1957.

32. Epstein, M. A.: Observations on the Rous virus; purification and identification of the particles from solid tumors. *Brit. J. Cancer*, 12: 248–255, 1958.

33. Evans, R. W.: *Histological appearance of tumors.* With a consideration of their histogenesis and certain aspects of their clinical features and behavior. E. S. Livingstone, Ltd., Edinburgh and London, England, pp. 660–679, 1956.

34. Feldman, W. H.: *Neoplasms of domesticated animals.* W. B. Saunders Co., Philadelphia, 1932.

35. Furth, J.: Observations with a new transmissible strain of the leucosis (leucemia) of fowls. *J. Exper. Med.*, 53: 243–267, 1931.

36. Furth, J.: Erythroleukosis and the anemias of the fowl. *Arch. Path.*, 12: 1–30, 1931.

37. Gaylord, W. H.: Virus-like particles associated with Rous sarcoma as seen in sections of the tumor. *Cancer Research*, 15: 80–83, 1955.

38. Haddad, M. N., R. A. Bonar, G. S. Beaudreau, C. Becker, and J. W. Beard: Virus of avian myeloblastosis. XV. Structural loci of virus synthesis and of adenosine-triphosphatase activity by electron and light microscopy of myeloblasts from tissue culture. *J. Nat. Cancer Inst.*, 24: 971–994, 1960.

39. Haguenau, F.: Significance of ultrastructure in virus-induced tumors. *Nat. Cancer Inst. Monograph*, No. 4: 211–250, 1960.

40. Haguenau, F., A. J. Dalton, and J. B. Moloney: A preliminary report of electron microscopic and bioassay studies on the Rous sarcoma I virus. *J. Nat. Cancer Inst.*, 20: 633–650, 1958.

41. Haguenau, F., H. Febvre, and J. Arnoult: Ultrastructure du virus du sarcome de Rous *in vitro*. *Compt. rend Acad. Sc.*, 250: 1747–1749, 1960.

42. Hall, W. J., C. W. Bean, and M. Pollard: Transmission of fowl leucosis through chick embryos and young chicks. *Am. J. Vet. Res.*, 2: 272–279, 1941.

43. DeHarven, E., and C. Friend: Further electron microscope studies of a mouse leukemia induced by cell-free filtrates. *J. Biophys. & Biochem. Cytol.*, 7: 747–852, 1960.

44. Heine, U., G. S. Beaudreau, C. Becker, D. Beard, and J. W. Beard: Virus of avian erythroblastosis. VII. Ultrastructure of erythroblasts from the chicken and from tissue culture. *J. Nat. Cancer Inst.*, 26: 359–388, 1961.

45. Iwakata, S.: Electron microscopic observations of chicken erythroblastosis. II. Report with special reference to varied features of macrophages as reservoirs of the virus. *Ann. Report Inst. Virus Res.*, 1: 221–242, 1958.

46. Iwakata, S., and S. Amano: Causative virus of chicken erythroblastosis observed in ultra-thin sections under the electron microscope and the modus of intercellular proliferation of this virus. *Acta Haem. Jap.*, 21: 154–169, 1958.

47. Johnson, E. P.: Focal leukosis manifestations, transmissions and etiological relationships of various forms. *Va. Agr. Exper. Sta. Tech. Bull.*, 76: 3–21, 1941.

48. Lee, C. D., H. L. Wilcke, C. Murray, and E. W. Henderson: Fowl leukosis. *J. Infect. Dis.*, 61: 1–20, 1937.

49. Moore, D. H., E. Y. Lasfargues, M. R. Murray, C. E. Haagensen, and E. C. Pollard: Correlation of physical and biological properties of mouse mammary tumor agent. *J. Biophys. & Biochem. Cytol.*, 5: 85–92, 1959.

50. Oberling, C., W. Bernhard, and P. Vigier: The internal structure of the virus-like particles associated with the Rous sarcoma and their presence in other neoplastic and normal chicken tissues. *Nature*, 180: 386–389, 1957.

51. Oberling, C., and M. Guerin: Nouvelles recherches sur la production de tumeurs malignes avec le virus de la leucémie transmissible des poules. *Bull. Assoc. franc. pur l'étude due cancer*, 22: 326–360, 1933.

52. Olson, C., Jr.: A transmissible lymphoid tumor of the chicken. *Cancer Research*, 1: 384–392, 1941.

53. Parsons, D. F., G. S. Beaudreau, C. Becker, R. A. Bonar, and J. W. Beard: Cell multiplication, virus liberation and ultrastructure of avian myeloblasts in tissue culture. *Acta Unio Internat. contra cancrum*, 15: 826–831, 1959.

54. Parsons, D. F., J. C. Painter, G. S. Beaudreau, C. Becker, and J. W. Beard: Tissue culture and circulating myeloblasts of avian leukosis studied in electron micrographs of ultrathin sections. *Proc. Soc. Exper. Biol. & Med.*, 97: 839–844, 1958.

55. Simms, H. S., and M. Sanders: Use of serum ultrafiltrate in tissue cultures for studying deposition of fat and for propagation of viruses. *Arch. Path.*, 33: 619–635, 1942.

56. Stewart, S. E., and B. E. Eddy: Properties of a tumor-inducing virus recovered from mouse neoplasms. In: *Perspectives in Virology* (M. Pollard, ed.), John Wiley and Sons, Inc., New York, 1959, pp. 245–255.

57. Stubbs, E. L., and J. Furth: The relation of leukosis to sarcoma of chickens. I. Sarcoma and erythroleukosis (strain 13). *J. Exper. Med.*, 61: 393–615, 1935.

58. Thorell, B.: Modifications of the host reaction towards leukemia virus. An experiment with virus-induced leukemia and kidney tumor in the chicken. *Proc. 7th Intern. Congress of Intern. Soc. Hematology*, Rome, 3: 582–592, 1958.

59. Thorell, B., and E. Yamada: The distribution of adenosinetriphosphatase activity in virus-induced leukemia cells. *Biochem. & Biophys. Acta*, 31: 104–114, 1959.

60. Troisier, J.: Leucose et sarcomatose des poules unite de virus. *Ann. Inst. Pasteur*, 55: 501–517, 1935.

61. Walter, W. B., B. R. Burmester, and C. H. Cunningham: *A study of the transmission of a viral-induced nephroblastoma (embryonal nephroma).* Am. J. of Vet. Res., in press.

62. Waters, N. F.: Breeding for resistance and susceptibility to avian lymphomatosis. *Poultry Sc.*, 24: 259–264, 1945.

63. Waters, N. F.: Mortality from lymphomatosis and other causes among inbred lines of white leghorns. *Poultry Sc.*, 30: 531–545, 1951.

64. Watson, M. L.: Staining of tissue sections for electron microscopy with heavy metals. *J. Biophys. & Biochem. Cytol.*, 4: 475–478, 1958.

65. Weinstein, D., J. R. Sommer, G. S. Beaudreau, C. Becker, and J. W. Beard: *Virus of avian myeloblastosis.* XVIII. Fixation of myeloblasts and phosphatase activity of loci of virus synthesis (viroplasts). J. Nat. Cancer Inst., 25: 1421–1449, 1960.

66. Uhl, E., J. Engelbreth-Holm, and A. R. Meyer: On the production of sarcomata in pure chicken leucosis strains. *Acta Path. et microbiol. scandinav.*, 13: 434–452, 1936.

FIG. 1. Electron micrograph of a thin section of a cell from primary nephroblastoma induced by the myeloblastosis BAI-A strain virus. Numerous extracellular virus particles (VP) may be seen. Nucleus (N) with invagination (1); the cytoplasm (CY) shows numerous mitochondria (M) and swollen endoplasmic reticulum (ER). Two arrows point to the "budding" process. × 16,000.

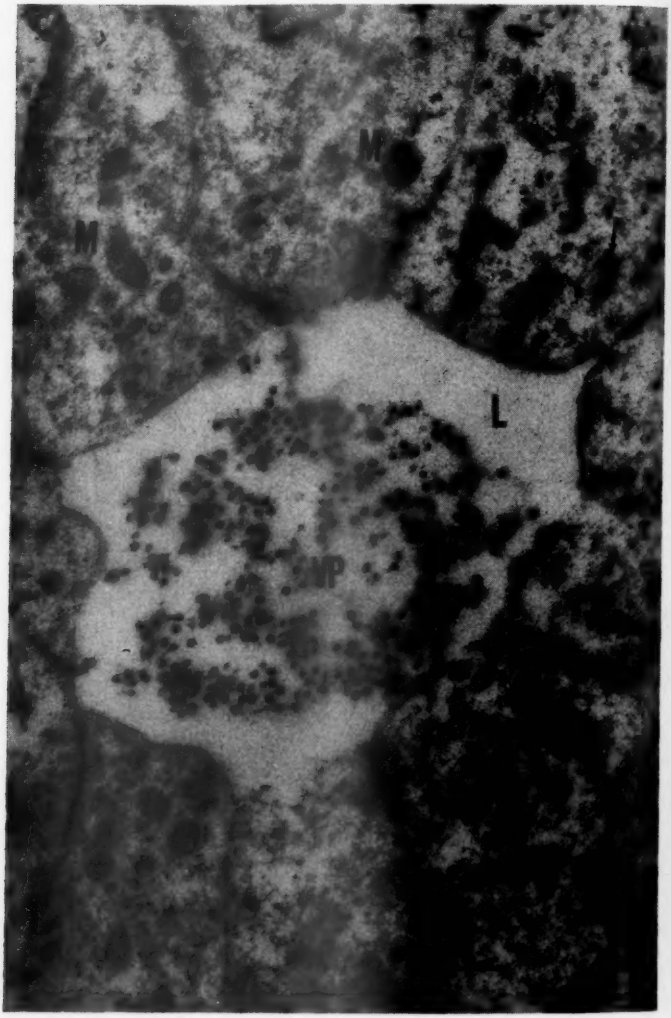


FIG. 2. Lumen (L) of a tubule in the transplanted nephroblastoma, showing a large group of virus particles (VP). The lumen is surrounded by cells, showing mitochondria (M), one containing a virus particle (arrow). × 20,000.

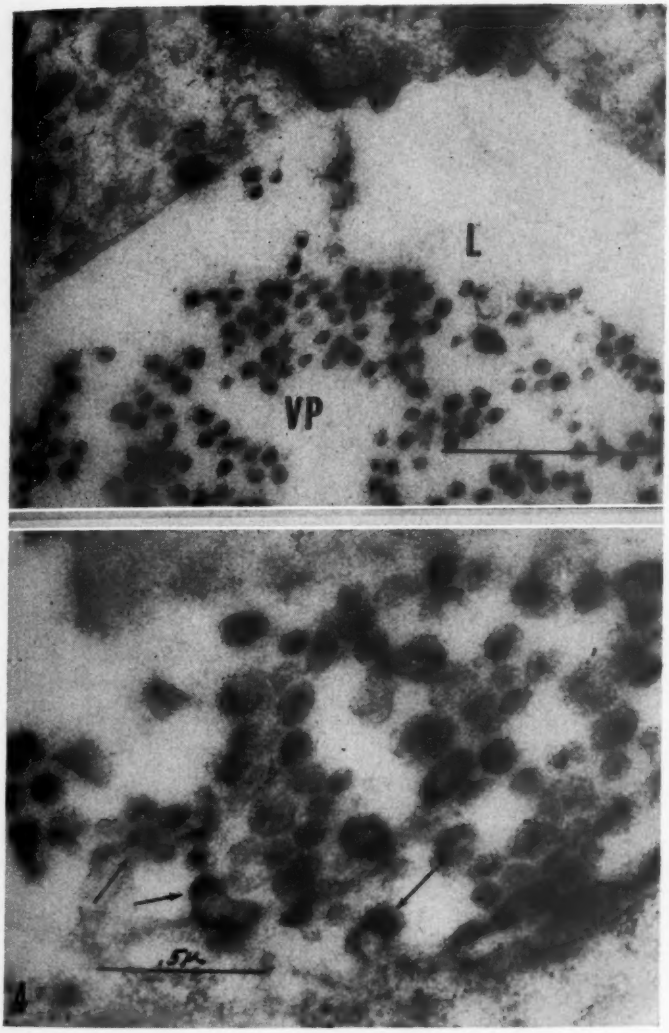


FIG. 3. Part of FIG. 2 at higher magnification. Internal structure of virus particles (VP) may be seen. × 40,000.

FIG. 4. "Budding" processes with apparent formation of virus particles by cellular membranes (arrows). Some virus particles show two concentric membranes and are devoid of the nucleoid. Primary nephroblastoma. × 68,000.

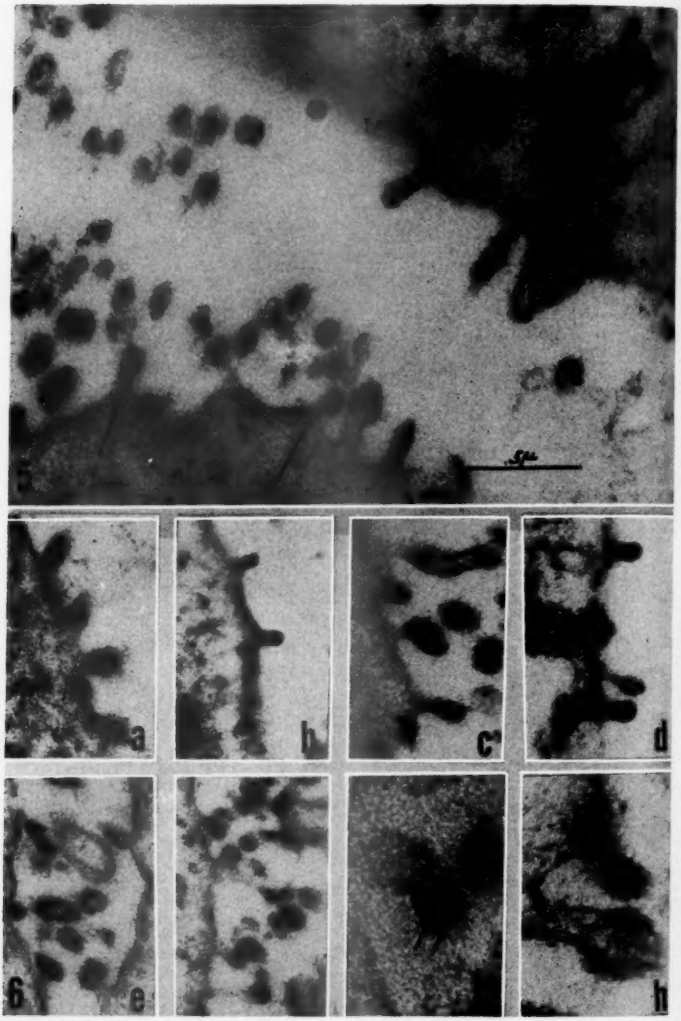
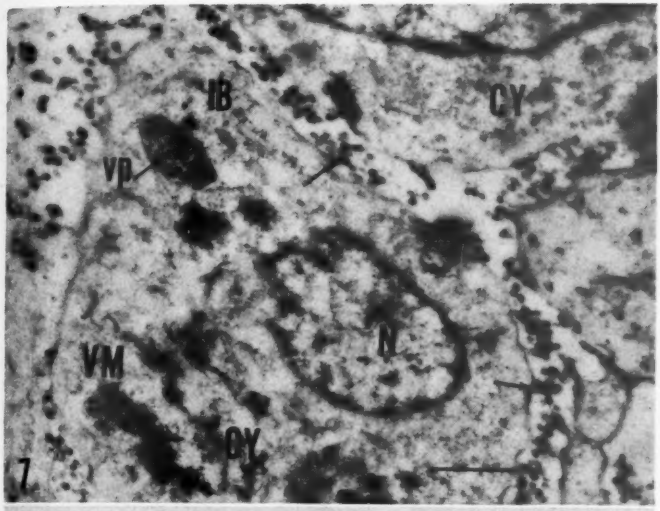


FIG. 5. Primary nephroblastoma. Part of two cells showing apparent formation of virus particles by cellular membranes (arrows). Virus particles in the intercellular space. × 48,000.

FIG. 6a-h. Examples of various stages of "budding" process by cellular membranes in primary and transplanted nephroblastoma.

FIGS. 6a-6f, × 42,000.

FIGS. 6g-6h, × 104,000.



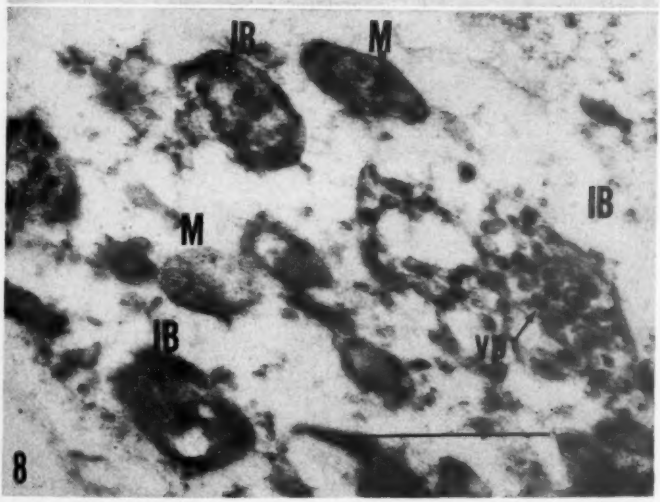


FIG. 7. Part of one cell of primary nephroblastoma, showing in the cytoplasm (CY) an early stage of viral matrix (VM) and an inclusion body (IB) of probable mitochondrial origin, filled with virus particles (VP). Some "budding" process is also present (arrows), nucleus (N) apparently unaltered. Numerous virus particles present in the intercellular spaces. × 16,000.

FIG. 8. Mitochondria (M) and inclusion bodies (IB) in the cytoplasm of a primary nephroblastoma. Virus particles (VP) may be seen in the inclusion bodies. × 45,000.

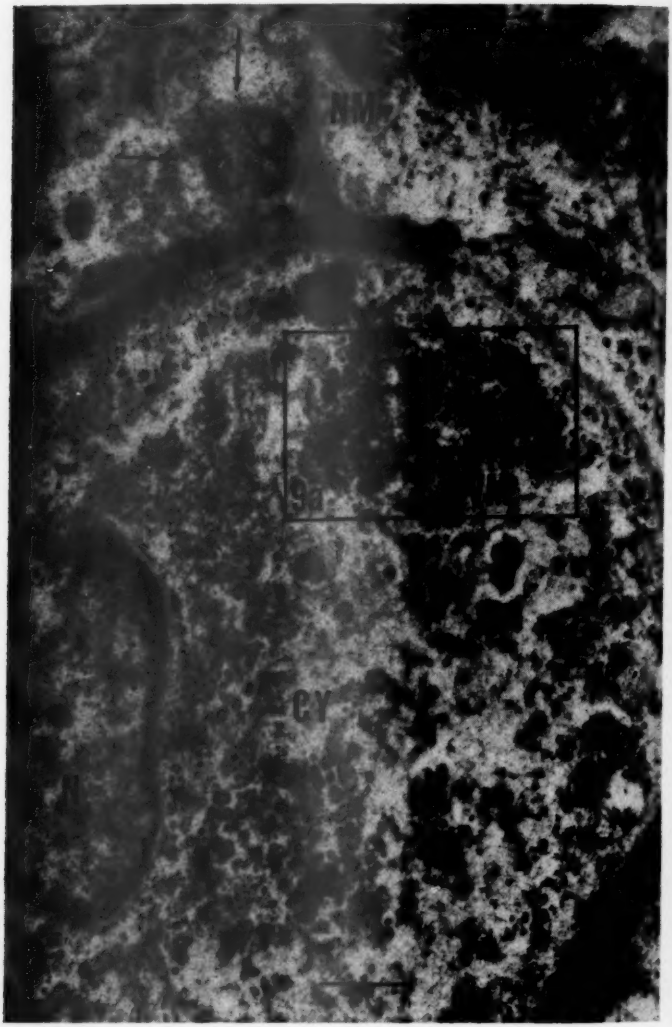


FIG. 9. Part of a cell of primary nephroblastoma, showing nucleus (N) with cytoplasm (CY) with viral matrix (VM). Part of the nucleus cut in oblique section, showing cross sections of channels in the nuclear membrane (NM) with material within the channels which appears as annuli (arrows). × 18,000.

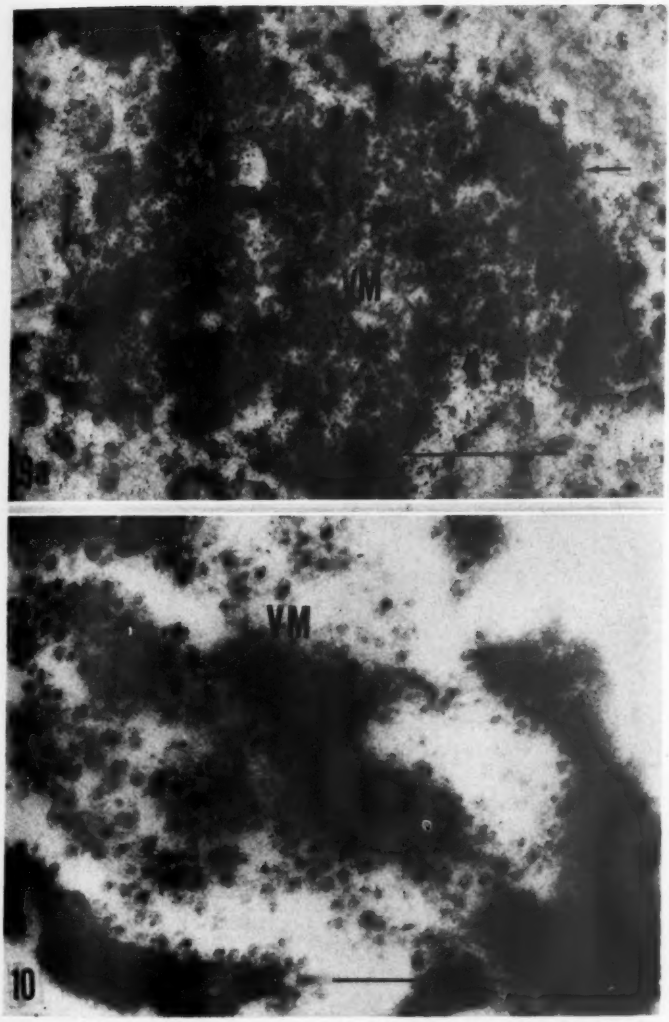


FIG. 9a. An enlarged area of FIG. 9, showing viral matrix (VM) at higher magnification. Arrows point out structures which may be early stages of virus particle formation. × 42,000.

FIG. 10. Viral matrix (VM) with structures which could be interpreted as virus particles in various stages of formation. Primary nephroblastoma. × 33,000.

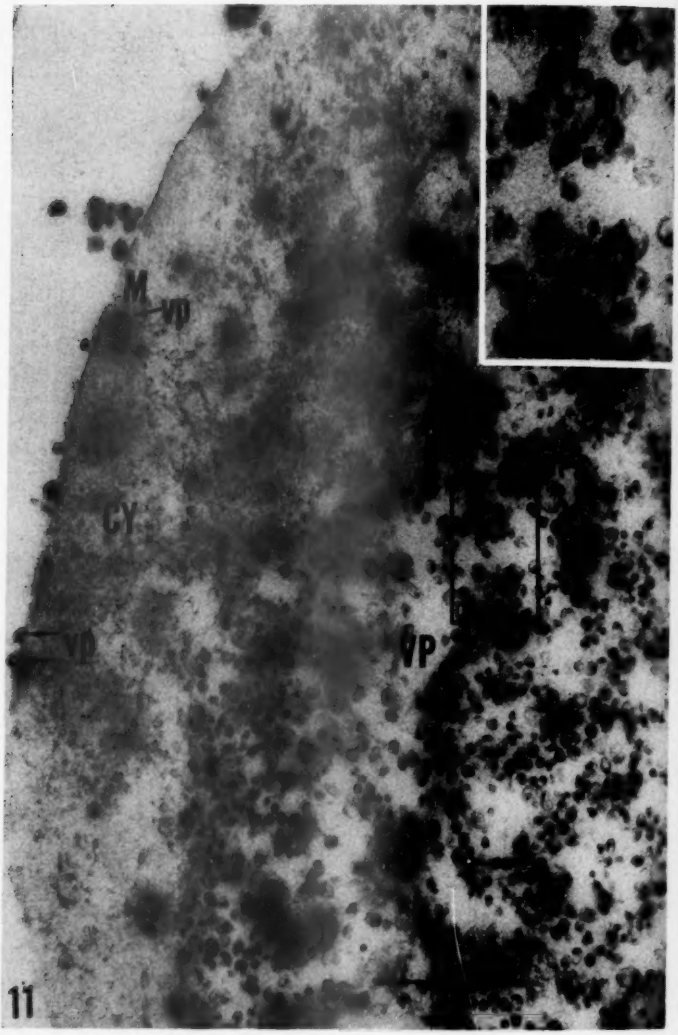


FIG. 11. Part of the cytoplasm (CY), showing virus particle (VP) in the final stages of formation from viral matrix. Mitochondrion (M) containing virus particle (VP). Virus particle (VP) formation by cellular membrane (arrows). × 30,000.

FIG. 11a. An enlargement of enclosed area of FIG. 11, showing virus particles of varying size and location of the nucleoid. × 56,000.

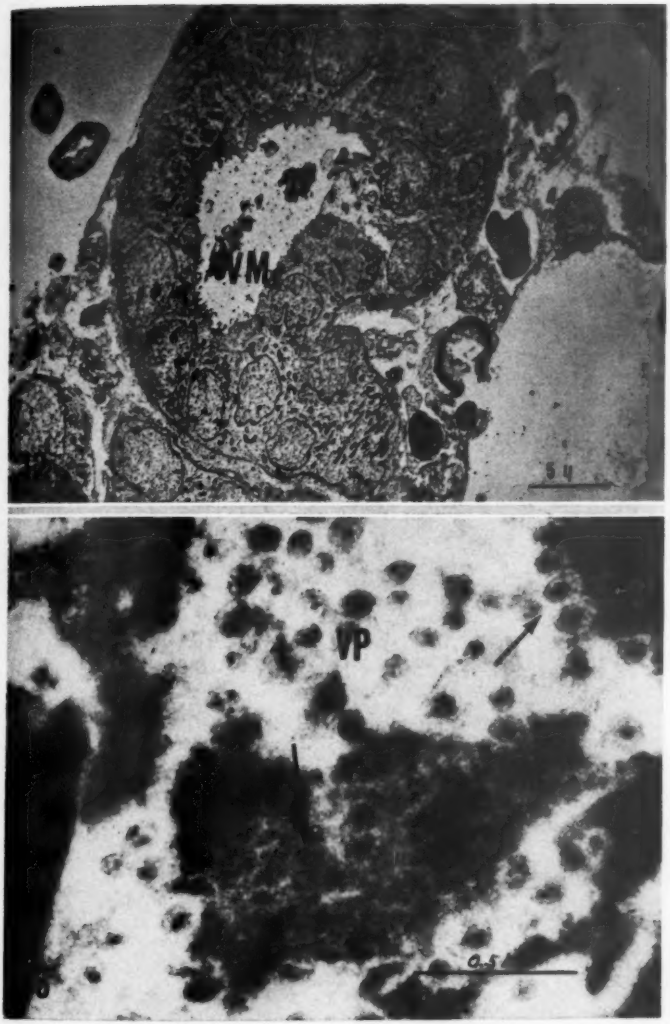


FIG. 12. Low power view of a tubule containing what appears to be viral matrix (VM) extruded into the lumen. Primary nephroblastoma. × 2,400.

FIG. 13. Part of viral matrix, shown in FIG. 12, at higher magnification. × 65,000.

FIG. 14. Low power view of spleen from a chicken with myeloblastosis BAI-A strain, showing areas containing virus particles (FIG. 14a). A mitotic figure (MF) may be seen. × 5,000.

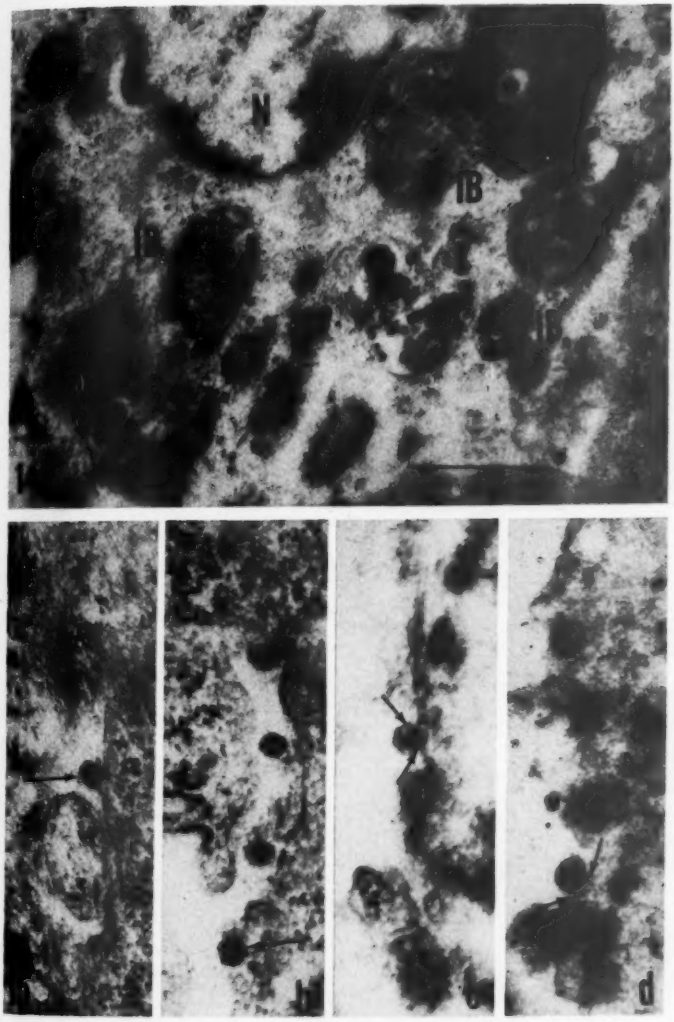


FIG. 14a. Part of FIG. 14 at higher magnification, showing cytoplasmic inclusion bodies of probably mitochondrial origin (IB) with virus particles. × 28,000.

FIG. 15a-d. Apparent virus particle formation by cell membranes. Cells of erythroid origin in the spleen of chickens with erythroblastosis (FIGS. 15a, 15b). Cells of myeloid origin in the bone marrow of chickens with myeloblastosis (FIGS. 15c, 15d). × 64,000.

FIG. 16. Cytoplasmic inclusion bodies of probable mitochondrial origin in the spleen of a chicken with myeloblastosis. × 40,000.

FIG. 17. Low power view of spleen from chicken with erythroblastosis strain R. × 5,000

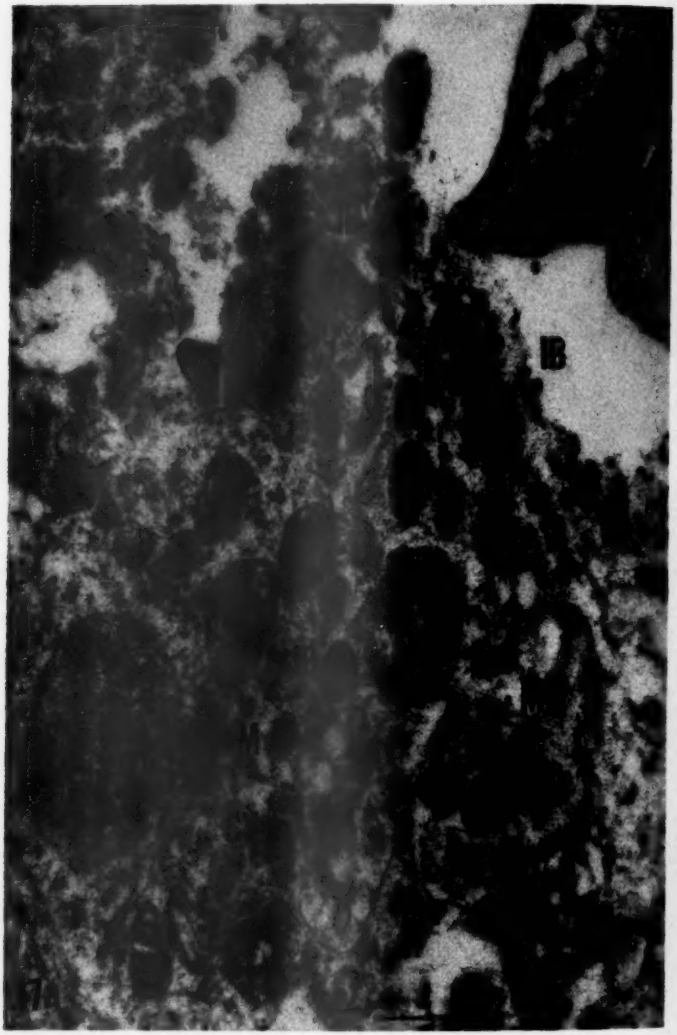


FIG. 17a. The area outlined in FIG. 17, at higher magnification. Mitochondria (M) and inclusion bodies presumably of mitochondrial origin (IB) containing virus particles may be seen. Membranous structures (MS). Part of the cytoplasm (CY) of a cell, probably a macrophage. × 24,000.

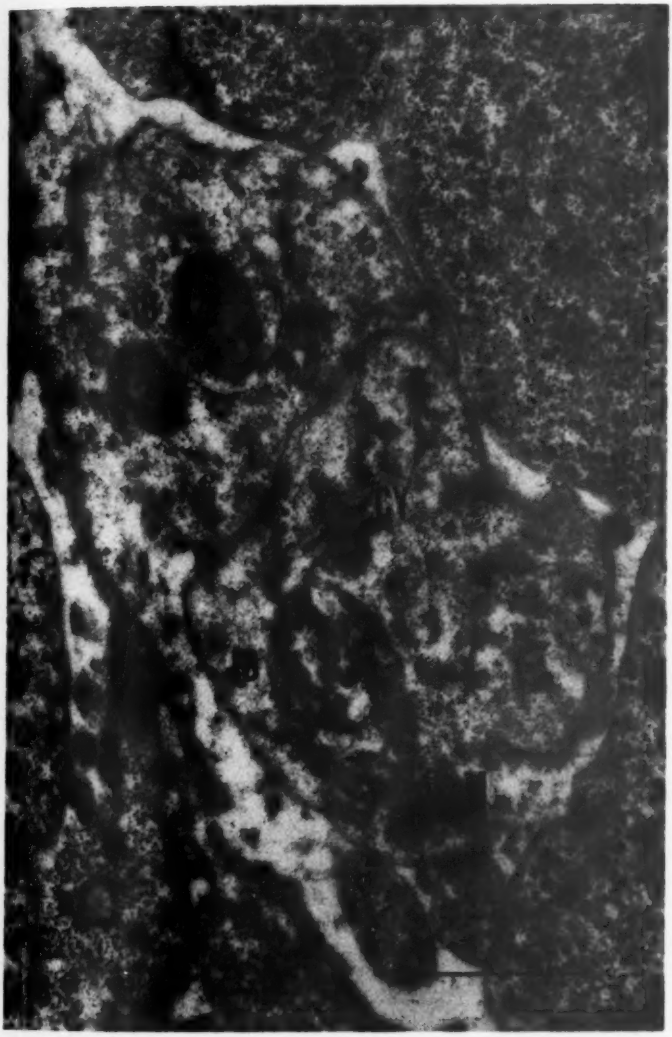


FIG. 18. Inclusion bodies (IB) of probable mitochondrial origin containing virus particles and mitochondria (M) in the cytoplasm of cells from spleen of chicken with erythroblastosis. × 36,000.

THE USE OF A MOUSE ORAL LD50 TO EVALUATE THE IMMUNOGENICITY OF *TRICHINELLA* METABOLIC ANTIGENS[a]

ADAM EWERT[b] AND LEROY J. OLSON[c]

In studies designed to induce immunological tolerance in the white mouse to *Trichinella spiralis* (Ewert and Olson, 1960), the problem of evaluating the immunogenicity of antigenic materials has been of vital importance. While the antigenic composition of *Trichinella* larvae is known to be very complex (review: Kagan, 1960; Olson *et al.*, 1960), several workers have directed their attention to *Trichinella* metabolites as immunogenic materials; the resistance of mice injected with these metabolites to sublethal infection has been measured by comparative worm burdens at necropsy (Campbell, 1955; Chipman, 1957; Chute, 1956; Ewert and Olson, 1960). This report presents results on the use of an oral LD50 challenge infection as a method of evaluating metabolic antigens hereafter called ES antigens (excretions and secretions of cultured larvae).

Materials and Methods

Fairfield-Webster white mice were used in this study. The stock infection of *Trichinella* was maintained by passage in Holtzman male rats. Larvae for maintenance of stock infections, infection of experimental mice and production of ES antigen were recovered by pepsin digestion of skeletal muscles from rats necropsied 6 to 12 weeks after infection with 3000 larvae. Larvae recovered from muscle digests were washed and suspended in a nutrient broth-gelatin solution. Samples of this suspension were taken to determine the number of viable organisms per unit volume; an adjustment of this concentration of larvae was made to deliver the required number of larvae per dose for infecting animals.

ES antigen was prepared from supernate recovered from cultured larvae. Cultures were prepared by recovering known numbers of viable larvae from nutrient broth-gelatin suspensions; the larvae were washed and resuspended at a concentration of 2500 larvae per ml of Simms-beef serum ultrafiltrate; 500 units of penicillin and 500 mcg of streptomycin were added per ml of Simms solution. Ten ml amounts of this suspension were incubated in cotton-stoppered 50 ml flasks for 5 days at which time the supernate from cultures was recovered, pooled and kept at —20°C until use. Cultures in which 50

[a] This study supported by National Science Foundation Grant G 7104. Submitted for publication March 30, 1961.

[b] Present address: Department of Tropical Medicine and Public Health, Tulane University School of Medicine, New Orleans.

[c] Department of Microbiology, The University of Texas Medical Branch, Galveston.

percent or more of the larvae present were non-viable after the 5 days were discarded. Larvae were kept at 37°C throughout the above recovery and incubation procedures (Ewert and Olson, 1960).

Results

A total of 94 male mice were used in two experiments. For each experiment infective larvae were suspended in a nutrient broth-gelatin solution and administered from a common pool to each mouse by stomach intubation; seven groups of mice were infected with doses ranging from 500 through 10,000 larvae per mouse. Following infection dead mice were collected daily and weighed; live mice were weighed at 2 day intervals during the 30 day observation period after infection. A total of 85 female mice were similarly treated in replicate experiments, except that the two groups of mice receiving 500 and 10,000 larvae per mouse were omitted due to the results obtained with male mice. All mice were 24 ± 4 grams at the time of infection. Results of all experiments are combined in Table 1.

The first death was noted on the second day after infection in the group receiving 10,000 larvae per mouse. Subsequent deaths were dose dependent; *i.e.*, mouse survival time increased as dosage decreased. The pattern of deaths for male and female mice was found to be similar with the majority of deaths occurring during the first 11 days postinfection (see Fig. 1: mice receiving 500 or 10,000 larvae not included).

The mouse oral LD 50 was calculated by plotting percentages of dead mice for each dose group on logarithmic-probit graph-paper (Miller and Tainter, 1944). Agreement between the LD50 for male and female mice was very close; lines plotted for each sex intersected at the 50 percent axis to give an LD50 of 1850 ± 175 larvae for both sexes.

Records of mean weights for each group of mice during the 30 day period of observation showed that weight loss was also dose dependent and most apparent in those mice receiving the greater number of larvae.

Survival of ES-injected mice following an oral LD50 challenge. Three-week old experimental mice were each started on a series of intraperitoneal injections of ES antigen; a total of 0.9 ml of ES antigen was given each mouse in 6 divided doses spaced at 3 day intervals. Control mice (litter mates of ES-injected mice) were similarly treated except that only the carrier solution for ES antigen, *i.e.*, Simms-beef serum ultrafiltrate and antibiotics,

TABLE 1

MOUSE MORTALITY FOLLOWING GRADED DOSES OF VIABLE TRICHINELLA LARVAE

Days post-infection	MALE MICE: No. larvae per mouse (no. mice per group)							FEMALE MICE: No. larvae per mouse (no. mice per group)				
	500 (8)	1000 (15)	1500 (14)	2000 (14)	2500 (14)	5000 (14)	10000 (14)	1000 (18)	1500 (20)	2000 (18)	2500 (19)	5000 (10)
1												
2							●					
3						●	●●●●● ●●●●●					
4					●	●● ●●	●				●	●● ●●
5					●	●● ●	●			●		●● ●
6						●				●	●	●
7					●● ●	●●				●●	●● ●	●
8			●	●	●●	●●				●● ●	●● ●	●
9			●	●●	●			●	●	●●	●● ●	
10			●	●●● ●●	●● ●	●			●	●	●●	
11				●	●●			●	●● ●	●●	●●	
12										●		
13			●									
14										●		
15								●	●	●		
16			●							●		
17					●			●				
18											●●	
19												
20											●	
21				●								
22				●●								
23		●●										
24			●●	●								
25												
26												
27												
28		●										
29			●								●	
30												
Survivors end of 30 days	△△△ △△	△△△△ △△△△ △△△	△△△△ △△△	△	none	none	△	△△△△ △△△△ △△	△△△△ △△△△ △△	△△	none	none

● Death
△ Survival

was injected. One week after the last injection, when the mean weight of these mice was 23.2 grams, an oral LD50 of 1850 infective larvae was given each mouse by stomach intubation. An observation period of 12 days postinfection was selected on the basis of the results above.

The results of this experiment are shown in Table 2. Out of a total of 61 ES-injected mice, 17 mice or 28 percent died during the

12 day observation period. By contrast 54 percent of 22 control mice died during this same period. The difference between these percentages of survival was found to be statistically significant (chi-square test, Snedecor, 1956). The 54 percent death rate for control mice in this experiment was in close agreement with the calculated LD50 obtained in the experiments above. ES-injected mice were also found to lose less weight (16 percent) as compared to control mice (24 percent) during the 12 day period.

Discussion

In these studies an oral LD50 of 1850 *Trichinella* larvae per mouse was obtained when the observation period was 12 days postinfection. On the basis of body weight, extrapolation of these results indicate an LD50 of 77 larvae per gram body weight, and an LD100 of 208 larvae/gbw for 24 gram male or female Fairfield-Webster mice. Various workers have reported on the infective and lethal doses of *Trichinella* larvae for a variety of mam-

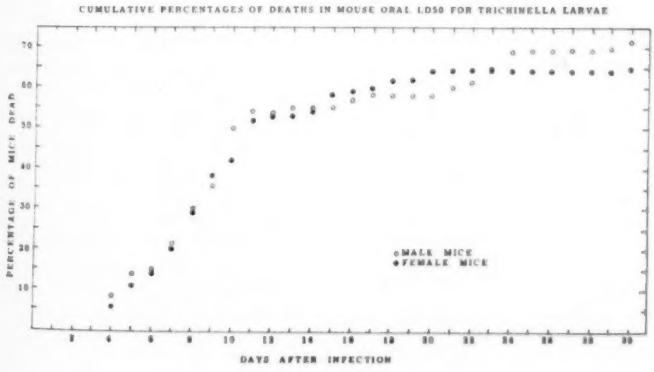
TABLE 2

Effect of prior immunization by intraperitoneal injections of mice with ES antigen on subsequent survival during a 12 day period after challenge with an oral LD50 of Trichinella *larvae*

Material injected	No. mice	Mean weight (grams) prior to infection	Percent weight loss	No. survivors	Percent deaths
ES	61	23.2	16	44	28*
Control	22	23.2	24	10	54

* Indicates difference between groups significant at the 5 percent level.

FIGURE 1


CUMULATIVE PERCENTAGES OF DEATHS IN MOUSE ORAL LD50 FOR TRICHINELLA LARVAE
PERCENTAGE OF MICE DEAD
DAYS AFTER INFECTION
oMALE MICE
•FEMALE MICE

malian hosts including mice, rats, hamsters, guinea pigs, monkey, dog, cow (review: Kagan, 1960; Zaiman *et al.*, 1961). Previous reports on the dose response of mice to *Trichinella* larvae have been concerned primarily with infective doses for immunity studies, and to a lesser extent with lethal doses; these latter experiments have been variable in terms of the host and the design, hence, comparative conclusions are very difficult. Zaiman *et al.* (1961) working with male Swiss albinos averaging 20–27 grams at infection reported a pattern of deaths over a 36 day observation period which correlated with dosage. The data of these workers suggests an LD50 which appears to be in general agreement with that reported in this paper.

Summary

1. The mouse oral LD50 for infective *Trichinella spiralis* larvae was found to be 1850 ± 175 for a 12 day observation period of both male and female mice weighing 24 ± 4 grams at challenge. A calculated LD50 of 77 larvae per gram of body weight and an LD100 of 208 larvae/gbw were obtained for mice under these conditions.

2. The immunogenicity of *Trichinella* larval metabolites was significantly demonstrated by the increased resistance of mice, sensitized by a series of intraperitoneal injections of larval metabolites, to an oral LD50 challenge of infective larvae.

REFERENCES

1. Campbell, C. H.: The antigenic role of the excretions and secretions of *Trichinella spiralis* in the production of immunity in mice. *J. Parasitol.*, 41: 483–491, 1955.
2. Chipman, P. B.: The antigenic role of the excretions and secretions of adult *Trichinella spiralis* in the production of immunity in mice. *J. Parasitol.*, 43: 593–598, 1957.
3. Chute, R. M.: The dual antibody response to experimental trichinosis. *Proc. Helminth. Soc. Wash.*, 23: 49–58, 1956.
4. Ewert, A., and L. J. Olson: Immunological tolerance studies with mice and *Trichinella*. *J. Parasitol.*, 46: 849–854, 1960.
5. Kagan, I. G.: Trichinosis: a review of biologic, serologic and immunologic aspects. *J. Inf. Dis.*, 107: 65–93, 1960.
6. Miller, L. C., and M. L. Tainter: Estimation of the LD50 and its error by means of logarithmic-probit graph paper. *Proc. Soc. Exp. Biol. Med.*, 57: 261–264, 1944.
7. Olson, L. J., B. Richards, and A. Ewert: Detection of metabolic antigens from nematode larvae by a microculture-agar gel technique. *Tex. Repts. Biol. Med.*, 18: 254–259, 1960.
8. Snedecor, G. W.: *Statistical Methods*. 5th ed. Iowa State College Press, Ames, Iowa, 1956.
9. Zaiman, H., R. G. Howard, and C. J. Miller: Mortality and survival of young male mice given single massive doses of *Trichinella spiralis* larvae. *Exp. Parasitol.*, 10: 206–210, 1960.

MIXED VIRAL INFECTIONS: INTERACTIONS BETWEEN COXSACKIE VIRUS AND POLIOVIRUS IN MICE AND TISSUE CULTURES[a]

SIDNEY HALPERN[b, c] AND S. EDWARD SULKIN[b]

The results of studies of mixed viral infections have found experimental application in diagnostic and definitive virology (20, 44), in suggested methods for rapid adaptation of viruses to desired milieu (30), and have stimulated debate on the possible effects of viral interactions in mass vaccination procedures and in epidemiologic studies of natural infections, especially among the enteroviruses (45).

Numerous factors may complicate the interpretation of data from mixed infections with Coxsackie virus and poliovirus. That these agents may propagate simultaneously in one host and even in the same tissue is suggested by the repeated observation that they may exist in the carrier state. The co-existence of these viruses has been demonstrated in clinical specimens from ill and well persons, in sewage, in flies trapped in poliomyelitis epidemic areas, in contacts of cases of poliomyelitis and in an intestinal carrier state in experimentally infected cynomolgus monkeys and chimpanzees (4, 25, 36, 54). In addition, it has been shown (37) that patients with paralytic poliomyelitis may excrete Coxsackie virus and poliovirus and respond with antibodies to both agents.

An epidemic of paralytic poliomyelitis characterized by a high incidence of dual infections with poliovirus and Coxsackie virus (32) provides support for an earlier suggestion (39) that certain

[a] Research supported in part by a grant from the Caruth Foundation of Dallas, Texas and by a pre-doctoral research fellowship from the National Institutes of Health. This paper contains the essential elements of a dissertation submitted by the senior author in partial fulfillment of the requirements for the Ph.D. Degree in December, 1960, at the University of Texas Southwestern Medical School. The authors wish to acknowledge the technical assistance of Miss Rae Allen and Mr. Craig Wallis. Submitted for publication April 6, 1961.

[b] From the Department of Microbiology, the University of Texas Southwestern Medical School, Dallas, Texas.

[b, c] Present address: Department of Epidemiology, Virus Laboratory, The University of Michigan School of Public Health, Ann Arbor, Michigan.

serologic types of Coxsackie virus may constitute a predisposing factor which might influence the clinical course of a poliomyelitis infection. More recently, experimental evidence has been presented (7) to support the hypothesis that central nervous system disease may at times depend upon a complex infection; a greater degree of pathology in monkeys resulted from mixed infections with Group A Coxsackie virus and poliovirus than from either agent alone. Dalldorf (5) and Stanley (52) reported that certain Group B Coxsackie viruses rendered mice resistant to poliomyelitis infection while this was not the case when Group A strains were used. No evidence of interference between Group A Coxsackie viruses and poliovirus could be demonstrated by Melnick (36), Levaditi and Vaisman (33), or Howitt and Nichols (25), and in many cases no influence could be shown on the course of experimental disease in monkeys or chimpanzees (1, 25, 36, 38, 52) except for the previously mentioned additive effect shown by Dalldorf and Weigand (7). Sulkin *et al.* (56) also showed that in poliovirus-infected adult mice prior inoculation of certain serotypes of Group B Coxsackie virus could exert a sparing effect on the course and outcome of the infection. The protection afforded was described in terms of both reduced mortality and altered symptomatology. The latter indications were recorded as changes in the progressive paralysis of mixedly infected animals as compared with control groups infected with poliovirus alone. Furthermore, one serotype of Coxsackie virus (B-2) produced disease in adult mice on subsequent inoculation of poliovirus. Thus, concomitant with an observable interference effect, heterologous virus exaltation also occurred. These experiments, which included variation in Coxsackie virus serotype and routes of inoculation, prompted the suggestion that preemption of certain cells is required for a primary virus inoculum to affect the pathogenesis of a challenge agent.

An understanding of the mechanisms involved in any of the phenomena resulting from mixed infections may be derived from the studies of the fates of each of the agents at the time of and following infection, and the specific changes induced in the infected cells. Implicit also for an understanding of these virus-host interactions is the necessity for accurate analysis of the quantitative aspects involved. Difficulties in making such determinations on infected cells in the intact animal include problems

of estimating the number of susceptible cells available and specifically engaged in the infectious process, the amount of virus actually entering susceptible cells, and the yields derived in a single growth cycle. These virus yields may be carried away by body fluids, or may be acted upon by various specific and nonspecific viral inhibitors. Experiments with uniform populations of cells would obviate some of these difficulties. In fact, some of the quantitative parameters associated with mixed viral infection in tissue culture systems have already been defined by such procedures (31, 35, 49). In the present studies an effort has been made to correlate the results of mixed infections with various serotypes of Coxsackie virus and the type 2 poliovirus in intact animals with those in cell cultures.

Materials and Methods

Animals. Newborn and three- to four-week-old Swiss albino mice (CFW) were used throughout these studies. Except where otherwise specified, all Coxsackie virus titrations were carried out in randomized newborn mice, and all poliovirus titrations in three- to four-week-old mice.

Viruses. Because of the differences among them (16, 53, 55), eight Coxsackie virus strains, representing seven serologic types, were used in these experiments. Three Group A Coxsackie viruses (A-1, A-2, and A-3) were supplied by Dr. Gilbert Dalldorf, and an A-4 and two Group B strains (B-1 and B-3) were provided by Dr. Joseph L. Melnick. Another A-4 strain (Dallas M.B.) was isolated in 1949 in this laboratory. A B-4 strain was supplied by Dr. F. S. Cheever. All Coxsackie viruses have been maintained by passage in infant Swiss albino mice. The Lansing strain of poliovirus, which has been maintained by intracerebral passage in white Swiss mice, was provided by Dr. Albert Milzer, and the MEF 1 strain was obtained from Dr. A. Howard Fieldsteel.

In preparing stock suspensions of the Lansing strain of poliovirus, several mice were infected by the intracerebral route and the cords and medullae from animals which succumbed to the infection within 5 days were harvested. A 20% suspension of the infected tissue was prepared in saline and stored in sealed glass ampoules at —20C. Each stock Coxsackie virus suspension consisted of 20% brain or carcass from infant mice which had become paralyzed following subcutaneous inoculation. All Coxsackie virus stock suspensions were treated with 2,000 units of penicillin and 10 mg of streptomycin per ml and supernatants following centrifugation at 2,000 rpm to 3,000 rpm for ten minutes were tested for bacterial contamination, dispensed in sterile glass ampoules, sealed and stored at —20C. Under these conditions the virus titer remained constant for several months. The method of Reed and Muench was used for calculating the LD_{50} titer, and that of Kärber for arriving at the 50% end-points ($TCID_{50}$) in tissue culture (29, 46).

Relatively pure genetic stocks of Coxsackie virus and poliovirus to be used in tissue culture experiments were obtained by serial passage at limiting dilutions or through the selection of isolated plaques on agar overlayed monolayers. The viruses were passed once in the cell line to be used before an experiment was initiated.

All animals were inoculated while under light ether anaesthesia, and throughout the text i.c. refers to intracerebral; i.p. intraperitoneal; i.s. intraspinal; i.m. intramuscular; and s.c. subcutaneous inoculations. When the intraspinal route of inoculation was used, the technique described by Habel and Li (17) was employed. Animals that died or were found dead within 24 hours after inoculation were excluded from the analyses and were considered to be deaths from trauma. Unless otherwise indicated, all animals were observed twice and sometimes three times daily during the observation period for extent of paralysis and deaths from poliovirus or Coxsackie virus infection.

Antisera. Type-specific antisera were prepared in rhesus monkeys by the methods of Salk *et al.* (50) and the technique of Miya *et al.* (42) was used for preparing mouse anti-viral ascitic fluid. In each case the virus stocks for use as antigens were prepared in monolayer cultures of monkey kidney cells, and these cells were used for this purpose only. One ml of the monkey antisera at 1:50 dilution for Coxsackie virus and poliovirus neutralized 3.3 and 4.2 $\log_{10}$ of each agent respectively; 1:10 dilution of the mouse ascitic fluid was found to neutralize 2.8 $\log_{10}$ of poliovirus and 2.4 $\log_{10}$ of Coxsackie virus. No cross-neutralization could be demonstrated with any of these antibody preparations.

Tissue cultures. Strains of the original line of HeLa cells and of the S3 clonal line were obtained from Microbiological Associates, Inc., and were grown to monolayers in 8 oz. prescription bottles from a seed inoculum of 300,000 cells per ml and in 15 × 125 mm screw capped tubes from 100,000 cells per ml. Cells in single-state suspension were prepared in several types of vessels. All cells were grown in a medium consisting of 0.5% lactalbumin hydrolysate in Earle's saline with 0.02 g% NaHCO3, supplemented with 30% horse serum, and containing 100 units of penicillin and 100 mg streptomycin per ml. An antibiotic mixture containing a final concentration of 80 units of polymixin B sulfate, 0.2 mg of neomycin, and 40 units of bacitracin per ml was alternated with the penicillin and streptomycin mixture in all growth media. The horse serum concentration was reduced to 10% when used as a maintenance medium for virus growth or titration, and the bicarbonate was adjusted to 2.2 g per L for these purposes. When plaque titrations were carried out the ingredients of the maintenance medium in 2X concentration were mixed with an equal volume of 2.8% agar (Difco) which was freshly prepared, autoclaved and kept liquid at 44C before use. All media were gassed with CO_2 to approximately neutrality before use. Throughout the text m.s. refers to maintenance medium; Ca-free m.s. refers to maintenance medium from which the $CaCl_2$ has been omitted; and g.m. to growth medium.

Results of In Vivo Experiments

The effects of Group A Coxsackie viruses on poliovirus pathogenesis in adult mice.—Group A Coxsackie viruses have been reported not to interfere with poliomyelitic infections of adult mice or monkeys (1, 5, 25, 36, 38, 52). Instead there have been indications that an enhancement of poliovirus-induced disease could be demonstrated in mixed infections with these agents (1, 7, 52). In an attempt to extend these earlier investigations to other types of Group A Coxsackie viruses five Group A Coxsackie viruses (A-1, A-2, A-3, and two A-4) were inoculated in separate experiments into different groups of four-week-old mice in massive multiple doses followed by injections of the type 2 (Lansing strain) poliovirus. Although the Coxsackie virus was administered by i.c. and i.p. routes, and at varying time intervals before the i.c. poliovirus injection, no interference with the course or outcome of the poliovirus disease was observed. In one experiment with an A-4 Coxsackie virus strain, however, an enhancement of poliovirus-induced mortality was suggested by a decreased incubation period. These results are in general accord with the work previously mentioned.

The effect of poliovirus on Coxsackie virus pathogenesis in infant mice.—Since immature mice are ordinarily susceptible to i.p. inoculation of Coxsackie virus, but not to poliovirus, experiments were designed to determine whether this general lack of effect of the Group A Coxsackie viruses on poliovirus-induced disease could be demonstrated as a reciprocal characteristic common to both agents. Accordingly, each of one group of three-day-old mice received an i.p. injection of 1,000 LD_{50} of the Coxsackie virus (A-4) and served as controls. A second group of infant mice each received two i.p. injections (100 LD_{50} each) of the type 2 poliovirus 20 and 18 hours prior to a challenge inoculation of 1,000 LD_{50} of Coxsackie virus, and one i.p. injection of poliovirus (100 LD_{50}) 4 hours after challenge. Each of a third group of mice received two i.p. injections of poliovirus (100 LD_{50} each) 20 and 18 hours prior to challenge with 1,000 LD_{50} of Coxsackie virus, but no further injection of poliovirus. The final group of mice each received three i.p. injections of poliovirus (100 LD_{50} each) at 0, 2 and 22 hours. Animals were observed twice daily

during the 10 days following the Coxsackie virus injections. These results are summarized in Table 1. A sparing effect by the poliovirus on both the course and outcome of infection with Coxsackie virus may be seen. By the fourth day approximately ⅓ of the animals inoculated with poliovirus in addition to Coxsackie virus had succumbed as compared with 78% deaths of animals inoculated with Coxsackie virus alone. However, at the termination of the experiment, although all of the control animals had died, approximately 15 and 28% of the second and third groups, respectively, were still alive. None of the mice receiving massive doses of poliovirus alone showed evidence of infection. As a further control, groups of infant mice were injected with 0.05 ml of a 1% suspension of normal adult mouse brain 20 and 18 hours prior to a challenge inoculation of 1,000 LD_{50} of Coxsackie virus and a single injection 4 hours after challenge. Since no significant difference in onset of symptoms or death was observed during the 10 day observation period, it is hardly likely that the sparing effect noted was due to an inflammatory process resulting from the injected brain tissue as had been suggested in other interfering systems (6, 57).

TABLE 1

Interference between poliovirus (type 2) and Coxsackie virus (A-4) in 3-day-old suckling white Swiss mice

Treatment		Group I Coxsackie virus*	Group II Type 2 poliovirus† and Coxsackie virus*	Group III Type 2 poliovirus‡ and Coxsackie virus*	Group IV Type 2 poliovirus§
Number of mice		24	26	29	17
Cumulative per cent deaths from Coxsackie virus infection (days after injection of Coxsackie virus)	3	20.9	0	3.5	0
	4	77.8	34.6	37.9	0
	5	88.9	65.4	55.2	0
	6	100.0	80.8	68.9	0
	7	100.0	80.8	72.4	0
	8	100.0	80.8	72.4	0
	9	100.0	84.7	72.4	0
	10	100.0	84.7	72.4	0
Survivors, per cent		0	15.3	27.6	100.0

* 1,000 LD_{50} doses injected intraperitoneally.

† Two intraperitoneal injections (0.1×10^{-3} ml. each) 20 and 18 hours prior to challenge inoculation of Coxsackie virus and one injection 4 hours later.

‡ Two intraperitoneal injections (0.1×10^{-3} ml. each) 20 and 18 hours prior to challenge inoculation of Coxsackie virus.

§ Three intraperitoneal injections (0.1×10^{-3} ml. each) at intervals of 0, 2 and 22 hours.

To establish whether or not the poliovirus was exerting its effect on Coxsackie virus proliferation, an experiment was carried out to determine the effects of poliovirus injection on the titer of the Coxsackie virus during the course of the experimental mixed infection. LD_{50} Coxsackie virus titrations were made in each of three groups of three-day-old mice. Each of one group received two i.p. injections (100 LD_{50} each) of type 2 poliovirus 24 and 18 hours prior to the inoculation of Coxsackie virus. The second group of mice each received Coxsackie virus alone, and each animal in the third group received normal adult mouse brain (0.05 ml of a 1% suspension) instead of the poliovirus prior to Coxsackie virus inoculation. The Coxsackie virus was inoculated i.p. into ten to twenty mice of each group at dilutions ranging from 10^{-3} to 10^{-7}. Calculations of LD_{50} titers were made beginning the third day after Coxsackie virus inoculation and at 12 hour intervals thereafter, for 15 days. As in previous experiments the sparing effect of the poliovirus on the early Coxsackie virus-induced mortality was notable. By the fourth day there was almost 2 $\log_{10}$ decrease in the LD_{50} titer in mice receiving both viruses as compared with those receiving Coxsackie virus alone. This difference became less conspicuous as the experiment progressed, and when calculations were made 15 days after the experiment was initiated the LD_{50} titers of all groups were essentially the same.

The effect of Group B Coxsackie viruses on poliovirus pathogenesis in adult mice.—Although the Group A Coxsackie viruses employed produce generalized myopathy in suckling mice with little, if any, evidence of damage to the central nervous system, the reason for utilizing these agents in the experiments just described stemmed from the work of Vilches and Hirst (58) who had shown that interference might occur among viruses with different tissue tropisms. More profound effects, however, have been observed when the interfering and the challenge viruses have similar sites of predilection (19, 51). Therefore, it seemed likely that an extension of the work with the Group B Coxsackie viruses, which produce lesions of the central nervous system in addition to focal myopathy, might show greater influence on the course of experimental infection of adult mice with type 2 poliovirus. Since timing and dosage play important roles in the out-

come of mixed infections, an effort was made to give the proposed interfering virus an advantage over the challenge agent.

In the first series of experiments separate groups of three- to four-week-old mice were inoculated i.c. with massive doses of Coxsackie viruses (B-1 and B-4) and were challenged five days later with an i.c. injection of poliovirus. The results of earlier studies in this laboratory (7) with Coxsackie viruses (B-2 and B-3) showed marked alteration in the progress of poliomyelitis in mice, but no such effect was observed with these serotypes of Group B Coxsackie virus. Even when the experiment was repeated with 10 LD_{50} of poliovirus instead of the 150 LD_{50} originally employed the results were essentially unchanged. The susceptibility of three- to four-week-old mice to poliovirus infection was also unaffected by previous inoculation with Coxsackie virus (B-1) one hour, one day, or 18 days prior to challenge.

These results are in agreement with those of Melnick (36) who also was unable to demonstrate interference between a Coxsackie virus (B-1) and the Lansing strain of poliovirus. Dalldorf (5), on the other hand, reported that five to eight-day-old mice inoculated i.p. or s.c. with the same serologic type of Coxsackie virus were rendered resistant to type 2 poliovirus given i.c. 4 to 10 days later. These experiences could not be reproduced in this laboratory primarily because some of the five- to six-day-old mice showed evidence of Coxsackie virus infection and were moribund by the time they were to be challenged with poliovirus after an interval of 8 to 10 days. Furthermore, it is known that the susceptibility of mice to these viruses varies with the age of the animal; immature mice are less susceptible than adults to poliovirus while the reverse is true in the case of the Coxsackie viruses (4, 16, 36, 53, 55). That the sparing effect observed by Dalldorf (5) might have been due in part at least to the increased resistance of young mice to poliovirus infection is a conclusion supported by the work of Domok (11, 12, 13) who was able to alter the susceptibility of poliovirus-infected mice with prior inoculation of Coxsackie virus (B-1) by employing young adult animals.

Since it had been previously shown (56) that Coxsackie viruses (B-2 and B-3) could limit the progress of poliomyelitis in adult mice, and this effect could be seen with both i.c. and i.s. injections of Coxsackie virus (B-3) on subsequent poliovirus infection by the i.c. route, experiments were designed to determine the

effects of other routes, as well, on poliovirus pathogenesis. In the first series of studies each of one group of three-week-old mice was inoculated i.c. with 1,000,000 LD_{50} of Coxsackie virus (B-3) six days prior to injection i.c. with 150 LD_{50} of type 2 poliovirus. A second group of mice each received i.c. 0.05 ml of a 1% suspension of normal infant mouse carcass tissue in lieu of the Coxsackie virus, and was also injected with 150 LD_{50} of poliovirus six days later. From these experiments it was found that the course and character of the mixed infection differed markedly from that in which poliovirus was used alone. The survival rates, however, and differences in forelimb to hindlimb ratios did not vary to the same extent as had been observed in the earlier report (56).

In the second series of studies an effort was made to determine whether prior i.p. inoculation of Coxsackie virus would exert a similar sparing effect on poliovirus pathogenesis, and, in turn, whether the subsequent i.c. poliovirus inoculum would affect the activity of the Coxsackie virus. Accordingly, one group of three-week-old mice each was injected i.p. with 500,000,000 LD_{50} of Coxsackie virus (B-3) seven days prior to an i.c. injection of 150 LD_{50} of type 2 poliovirus. Another group of mice each received 0.05 ml of a 1% suspension of normal infant mouse carcass tissue instead of the Coxsackie virus and these animals were also inoculated seven days later with 150 LD_{50} of poliovirus. Brain-cord suspensions from animals which had died without previous evidence of paralysis and which had received both viruses were tested for Coxsackie virus by i.p. inoculation of newborn mice, and for poliovirus by i.c. inoculation of three-week-old mice. Deaths were considered to be due to Coxsackie virus in animals without evidence of paralysis if a 10^{-3} suspension of brain-cord was infective for suckling mice following i.p. inoculation and failed to produce infection in adult mice following i.c. inoculation of a 10% suspension. In addition, in most instances it was possible to distinguish between the two illnesses; the symptoms of infection due to Group B Coxsackie viruses consisted of roughened coat, hunching, tremors, and spasticity, while in the case of the poliovirus infection, aside from the flaccid paralysis in one or more limbs, the animals have smooth coats and appear otherwise healthy. The results of this experiment, summarized in Table 2, indicate that a sparing effect on polio-

TABLE 2

Effects in mice of intraperitoneal inoculation of Coxsackie virus (B-3) on subsequent intracerebral inoculation of poliovirus

Number of mice		70				63		
Procedure		Coxsackie virus and poliovirus¶				Normal infant mouse carcass Tissue and poliovirus§		
		Poliomyelitis deaths*		Deaths without paralysis*		Poliomyelitis deaths		
Results		Number	Following paralysis	Poliomyelitis	Coxsackie	Number	Following paralysis	Without paralysis
	3	9(12.8)	3	6†	.	12(19.0)	6	6
Animals infected and	4	7(22.8)	6‡	1	2	14(42.3)	13	1
	5	5(30.0)	4	1	2	6(50.8)	6	.
dying from	6	2(32.8)	2‡	.	.	2(53.9)	2	.
poliomyelitis	7	1(34.3)	1†	.	1	2(57.1)	2	.
or Coxsackie	8	2(37.1)	2‡	.	1	1(58.7)	1	.
virus infection	9	2(40.0)	2	.	1	0(58.7)	.	.
(days after	10	1(41.4)	1†	.	.	1(60.3)	1	.
injection	11–13	3(45.7)	2	1	.	9(74.6)	7	2
of Polio-	14–16	2(48.5)	0	2	.	4(80.9)	4	.
virus)	17–24	0(48.5)	0	.	.	2(84.1)	1	1
	25–30	1(50.0)	1†	.	.	1(85.7)	1	.
Survivors		50.0%				14.3% (p=.001)		

* Brain-cord suspension tested for Coxsackie virus in suckling mice (s.c.) and poliovirus in 3-week-old mice (i.c.).
† Not tested for either virus.
‡ Both viruses demonstrated in brain-cord suspension from one of these mice.
§ One i.p. injection of normal infant mouse carcass tissue instead of Coxsackie virus.
¶ One i.p. injection of Coxsackie virus (500,000 LD_{50}) 7 days prior to i.c. injection of 150 LD_{50} of poliovirus.

virus infection was exerted by the primary i.p. Coxsackie virus inoculation. Furthermore, in animals receiving both viruses the Coxsackie virus migrated to and multiplied in the central nervous system to sufficient levels to kill the animals.

It seems clear from the results of these *in vivo* experiments that alteration in route of inoculation and viral serotype in mixed infection with Coxsackie virus and poliovirus may give markedly varying effects on the course and outcome of the experimental infection. The fact that Coxsackie virus when injected into the same or higher levels of the central nervous system than the poliovirus produced lesser effects on subsequent poliovirus pathogenesis than when injected into lower regions, tends to substantiate the earlier conclusion (56) that pre-emption of certain sites by one virus is requisite for alteration of the activities of the other.

That these effects may be reciprocal is indicated by enhancement of Coxsackie virus activity in the same animals in which poliovirus disease had been suppressed. Domok (13) suggested that the significance of such studies lies in the possibility that the results might be of practical value in relation to human pathology. It is hoped that pathology in its broadest sense is implied because of the obvious implications that such investigations have in epidemiology, immunology, taxonomy, and ecology to name a few of the disciplines which may also derive either practical or theoretical information from similar data. The adaptation of cell culture technique to the examination of mixed viral infection together with the recent discoveries of extraviral substances (26, 27) which participate in certain interference effects seems to have evoked a general renewal of effort towards the elucidation of underlying mechanisms responsible for these reactions. It therefore seemed desirable to re-examine the effects of mixed infections with Coxsackie virus and poliovirus with more critically analytical methods provided by cell culture systems.

Results of In Vitro Experiments

The effects of Coxsackie virus (B-3) on the cytopathogenesis of type 2 poliovirus in HeLa cell monolayers. Since it is clear from the *in vivo* studies described above that a sparing effect could be demonstrated by the blocking action of Coxsackie virus (B-3) on the early mortality and progress of paralysis in mice subsequently infected with a type 2 (Lansing) poliovirus, an attempt was made to determine if a delay or diminution of cytopathogenesis by the MEF 1 (type 2) strain of poliovirus could be demonstrated in HeLa cell monolayers previously inoculated with a Coxsackie virus (B-3). Accordingly, tube monolayers of HeLa cells which had grown to complete sheets in 2 days from an original seed of 100,000 cells per ml were inoculated with dilutions of Coxsackie virus ranging from 10^{-4} through 10^{-7}. After incubation for 24 hours at 37° C groups of five tubes each were emptied of fluid and challenged with varying dilutions (10^{-3}, 10^{-4} and 10^{-5}) of poliovirus in m.s., in a 10^{-1} dilution of Coxsackie virus immune serum, or were replenished with m.s. alone. Parallel sets of five tubes each were also emptied of g.m. on day 0, replenished with m.s. and treated 24 hours later as though they had received a primary virus inoculum. All tubes were observed

at the same time daily for degenerative changes over a period of seven days after primary inoculation. HeLa cells in this system showed little, if any, increased resistance to poliovirus cytopathogenesis as the result of prior exposure to Coxsackie virus. The only indication of a delay in the development of cytopathic changes was observed in those tubes which had received the highest dilutions of both agents; in this case, a 10^{-7} dilution of Coxsackie virus which itself caused no degeneration seemed to extend the survival of monolayers also inoculated with a 10^{-5} dilution of poliovirus, an average of 0.6 days beyond the survival of cells with the same dose of poliovirus alone.

Effects of u.v. inactivated Coxsackie virus and poliovirus on heterologous virus cytopathogenesis in HeLa cell monolayers. An obvious difficulty in the interpretation of the results of experimental mixed viral infections is that a diminution of activity of the challenge agent may be obscured by the degenerative activities of the primary viral inoculum. Since immune serum shows no apparent inhibition of the Coxsackie virus once the agent is established in the culture, it seemed desirable to use another procedure likely to limit degenerative changes resulting from the primary inoculum while retaining whatever reactivity it might have with the challenge virus. Ultraviolet irradiation serves this purpose.

Using doses of u.v. irradiation sufficient to completely neutralize infectivity (poliovirus: 18 minutes, Coxsackie virus: 23 minutes) experiments were carried out to determine if u.v. inactivated virus could affect the cytopathogenesis of heterologous active virus. Varying dilutions of inactivated viruses were inoculated into groups of five monolayer tubes of HeLa cells each and incubated at 37° C for the time intervals indicated in Table 3. The tubes were then emptied of fluid and challenged with dilutions of active heterologous virus and reincubated at 37 C for an additional 24 hours. The tubes were again emptied, replenished with m.s. and reincubated. Observations were made daily for seven days and the results summarized in Table 3 are recorded as the average day of marked degeneration for five tubes. Those infected cultures showing no degenerative changes were arbitrarily given a figure of "seven" days for inclusion in the averages. Therefore, any effect by inactivated virus on the cytopathogenesis of the active agent may be discerned as either a higher or

TABLE 3

Effects of ultraviolet inactivated virus on active heterologous cytopathogenesis in HeLa cell monolayer cultures

	20 hours between u.v. poliovirus and active Coxsackie virus						19 hours between u.v. Coxsackie virus and active poliovirus					
Coxsackie dilutions	Dilutions of inactivated poliovirus				m.s.†	Poliovirus dilutions	Dilutions of inactivated Coxsackie virus				m.s.	
	10^{-1}	10^{-2}	10^{-3}	10^{-4}			10^{-1}	10^{-2}	10^{-3}	10^{-4}		
10^{-5}	2.6*	3.2	2.8	3.0	2.8	10^{-4}	2.2	2.4	2.6	2.2	2.4	
10^{-6}	4.2	4.4	4.6	4.4	4.4	10^{-5}	3.4	3.4	4.0	4.0	3.8	
10^{-7}	6.2	6.2	6.0	6.6	6.2	10^{-6}	4.8	4.8	5.4	5.6	6.8	
10^{-8}	7.0	7.0	7.0	7.0	7.0	10^{-7}	4.8	5.2	7.0	7.0	7.0	
m.s.†	7.0	7.0	7.0	7.0	7.0	m.s.	7.0	7.0	7.0	7.0	7.0	
	43 hours between u.v. poliovirus and active Coxsackie virus						41 hours between u.v. Coxsackie virus and active poliovirus					
10^{-5}	2.8	2.6	2.4	2.6	2.6	10^{-4}	2.6	2.0	2.4	2.2	2.4	
10^{-6}	4.2	4.6	4.6	4.2	4.6	10^{-5}	3.0	3.8	3.8	4.2	4.0	
10^{-7}	6.6	6.8	6.6	6.4	6.4	10^{-6}	4.6	4.6	5.4	5.4	6.6	
10^{-8}	7.0	7.0	7.0	7.0	7.0	10^{-7}	4.8	5.4	7.0	7.0	7.0	
m.s.	7.0	7.0	7.0	7.0	7.0	m.s.	4.8	7.0	7.0	7.0	7.0	

* Results are recorded as average day of marked cellular degeneration after active virus inoculation for five tubes.
† m.s. used instead of either active or inactivated viruses.

lower average day of degeneration than the control tubes which had received either one or the other virus; this average, however, could never exceed seven days. As a further control, tubes receiving only inactivated virus were included to cover the possibility of either incomplete inactivation of the primary inoculum or the occurrence of a homologous multiplicity reactivation.

The inactivated poliovirus caused no change in the average time of appearance of marked degeneration due to active Coxsackie virus. In those tubes which had primary inocula of inactive Coxsackie virus followed by high dilutions of active poliovirus there was a notable decrease in average time required for marked degeneration to occur. In some instances there was as much as a 2 day variation between the degeneration of monolayers exposed to both inactive Coxsackie virus and active poliovirus and those exposed only to active poliovirus.

While these studies were in progress a viral inhibitory substance, "interferon," produced by susceptible chick chorioallantoic cells in response to exposure to inactivated influenza virus, was reported to mediate interference among certain of the myxoviruses as well as between influenza virus and a variety of heterologous agents (26, 27, 28). An effort was made to de-

termine if a similar extraviral substance could imitate the effects of Coxsackie virus on poliovirus cytopathogenesis. Monolayers of HeLa cells were grown in 8 oz. prescription bottles and inoculated with 10^{-1} and 10^{-2} dilutions of Coxsackie virus irradiated for 23 minutes. Similar monolayers were exposed to m.s. which had been given the same dose of u.v. All bottles were then incubated at 37 C for 24, 48 and 72 hours, after which time the fluids were harvested, clarified by centrifugation at 1500 rpm for 10 minutes and frozen and stored at −20 C until used. The fluids from monolayers exposed to irradiated m.s. were referred to as "control" fluid, while those exposed to irradiated Coxsackie virus are designated "test" fluid. Groups of monolayer tube cultures of HeLa cells were exposed to "test" or "control" fluids and five tubes of each group were inoculated with each of a series of dilutions of active poliovirus ranging from 10^{-4} to 10^{-7} immediately, after 24 hours, and after 48 hours of incubation at 37 C. Twenty-four hours after each of the "test" and "control" fluid tubes had been in contact with virus they were emptied, replenished with m.s. and reincubated. Under these conditions there was no apparent difference between the average degeneration time of poliovirus infected monolayers pretreated with either "test" or "control" fluids, indicating that the Coxsackie virus particle itself acts directly to produce its effects on poliovirus cytopathology.

Studies of mixed viral infections of suspended cell cultures. Lack of sufficient sensitivity for the differentiation of interactions and for the determination of the effects on each of the host cells are important problems in analyses of the basic mechanisms operative in mixed viral infections. It therefore seemed conceivable that quantitative studies on the fate of each of the viruses alone, and in mixed infection, as well as on the survival of the host cells in susceptible populations might aid in the clarification of the seemingly enigmatic earlier results. Discussions of virus quantitation in monolayer cell culture systems suggest limitations in accuracy by mechanical trapping and readsorption of released virus, or by contiguous spread of intracellular virus (3, 15, 22, 23, 24). Furthermore determinations of host cell survival in virus-infected monolayers are handicapped by the requirement for a dispersing agent and mechanical manipulation which may also affect cell viability. To obviate these restrictions it seemed desirable to use suspended cell cultures.

Girardi, *et al.* (15) had shown that trypsinized HeLa cells, derived from monolayer cultures, could be kept viable for 24 hours in a single state suspension by means of a magnetic stirrer without significant loss in concentration as long as the glassware was coated with silicone to prevent adherence of cells to the vessel walls. These workers were able with this method to demonstrate cycles of multiplication of poliovirus and influenza virus similar to those of phage-bacterium systems, while also keeping account of the effects of the various phases of the growth cycles on the viability of host cells. In this manner the kinetics of virus replication were correlated with signs of cell death. It seemed likely that these techniques could provide a convenient device for studying the site or sites in the growth cycles of Coxsackie virus and poliovirus most sensitive to heterologous virus activity, while comparing the effects of single and mixed infection on the survival of host cells.

Monolayer bottle cultures of HeLa cells were dispersed with 0.25% trypsin in Ca-free m.s. with gentle pipetting. Cells were then centrifuged at 1500 rpm for 2 minutes, washed three times in Ca-free m.s. and suspended at a concentration of 150,000 cells per ml in complete m.s. containing the desired viral inocula. Such suspensions were then dispensed in 30 ml volumes into silicone-coated 25 x 150 mm screwcapped tubes containing a siliconed magnetic bar. Virus was allowed to adsorb to cells at 37 C for a two hour period with ten second stirring intervals every thirty minutes. Cells were then centrifuged, washed three times, resuspended in Ca-free m.s. and reincubated at 37 C. Samples were withdrawn periodically for virus determinations in cell and fluid phase fractions separated by centrifugation at 1500 rpm for 5 minutes, and for establishing the status of the host cell population. Virus titrations in mixed infections were carried out by incubating samples of mixed yields with each of the specific antisera at 37 C for 1 hour so that only the degenerative activity of the agent for which the antiserum was not specific could be measured. These titrations were made as end-point dilutions in tubes of HeLa cells after one cycle of freezing and thawing. All virus samples were kept frozen at −20 C until titrated. The viability of the cells was determined by mixing equal portions of cell suspension with 0.5% aqueous trypan blue and immediately counting in a hemocytometer. These cells which were imper-

meable to stain were considered viable. Figure 1 shows the results of infection in a suspension of cells at a concentration of $10^{5.2}$ per ml with a final poliovirus concentration of $10^{5.5}$ TCID50 per ml; curves were plotted as per cents of input of cells and virus against time after virus inoculation.

The delay in death of poliovirus-infected HeLa cells could be anticipated on the basis of the time required for adsorption, penetration and reorganization of cell synthetic mechanisms for the replication and maturation of virus. This has been noted by others (14, 34, 52) who have recorded cytologic degenerative changes associated with poliovirus proliferation activities. Essentially all of the virus was adsorbed by cells within 4½ hours since less than 1% could be detected in the suspending medium. The infected cells showed no marked degenerative changes until 6½ hours after addition of virus. At this time the first indications of cell death occurred and the cell-associated virus fraction reached a maximum. There was then a sharp increase in the number of cells with increased permeability accompanied by a drop in virus concentration detectable in the cell fraction. The

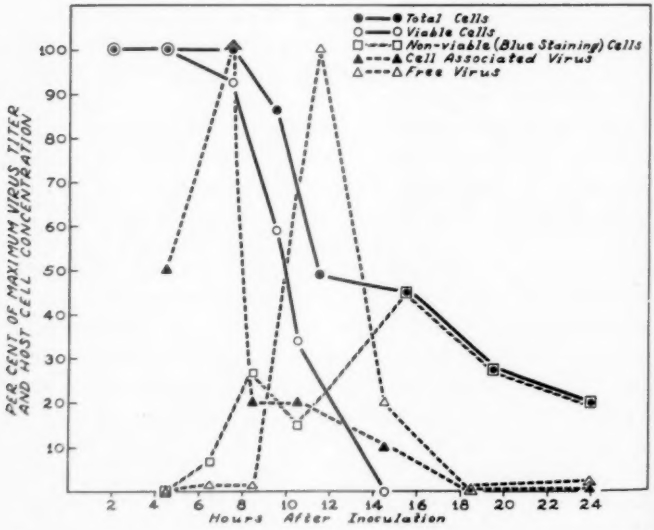


FIG. 1. Host cell (HeLa) survival and virus activities in type 2 (MEF_1) poliovirus-infected cell culture.

first increase of free virus did not occur until the cell-associated virus had dropped to 20% of its maxiumu. The subsequent rise of free virus to a maximum at 10½ hours after infection, simultaneous with a precipitous loss of both cell viability and cell integrity, is suggestive of a lytic-like process responsible for the liberation of polivirus from infected cells. The decline in cell-associated virus is probably due to the reduction of available cells since the same volume of suspension was used in all titrations. The subsequent loss of free virus, however, may be attributed to both thermal inactivation and/or adsorption to the remaining intact cells which were probably not able to replicate maximum viral yields due to loss of viability. Similar uninoculated cell suspensions showed no noticeable cytopathic effects during 48 hours of incubation. These cyclic patterns of poliovirus multiplication are in accord with results of others (14, 15, 22, 23, 34, 47).

The results observed in a suspended cell culture infected with a final concentration of $10^{6.5}$ TCID50 per ml of Coxsackie virus, and presented in Figure 2, show a similar pattern to that observed for poliovirus-infected cell populations although certain differ-

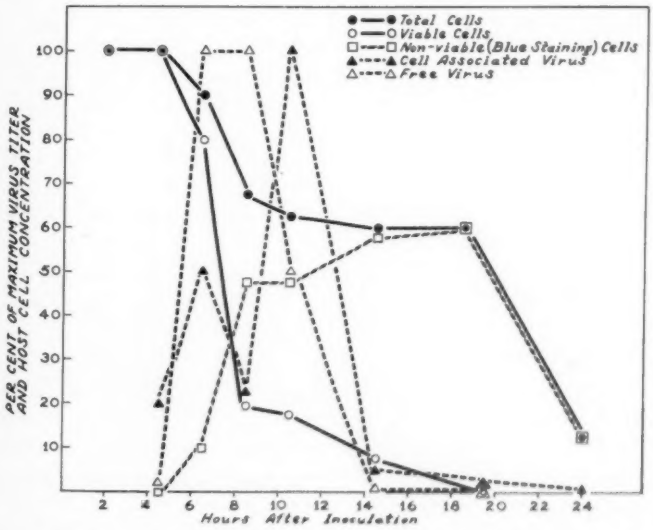


FIG. 2. Host cell (HeLa) survival and virus activities in type 3, Group B Coxsackie virus-infected suspended cell culture.

ences are apparent. Coxsackie virus induced a more precipitous early loss of cell viability; only 20% of the cells remained viable 8½ hours after inoculation at a time when 60% of the poliovirus infected cells were still alive. Insignificant amounts of both viruses remained unadsorbed 4½ hours after infection. The maximum free Coxsackie virus titer occurred at 6½ hours after infection and was accompanied by a rise in cell-associated virus to 50% of the inoculum. The primary decrease in cell-associated Coxsackie virus may also be attributed to the disintegration of cells since the same volume of cell suspension was used for all titrations. Although these data would suggest that a secondary, asynchronous cycle of multiplication might account for the decrease and subsequent increase of virus in this fraction, later experiments in which small enough cell numbers were used to preclude a secondary cycle indicated that this decrease followed a gradual and continuous rise to cell-associated virus maximum at 8½ hours after infection. The fact that the free Coxsackie virus titer was maintained at its maximum during this period would support the assumption that Coxsackie virus is released from cells at approximately the time of its maturation. Although poliovirus release was preceded by increased cell permeability followed by marked disintegration, these cytopathic events did not occur in Coxsackie virus-infected cells until after the release of virus into the medium. Differences between the results of infection of cells by these agents have also been demonstrated by other methods. Electron micrographs of Coxsackie virus-infected cells have shown only intracytoplasmic crystalline arrays, but no intranuclear involvement (43). Similar photographs for poliovirus as well as cytochemical studies indicate that both cell nucleus and cytoplasm are affected by this agent (47, 48). The latter properties have been further corroborated by fluorescent antibody studies (3, 18). On the basis of these various techniques it may be inferred that both overlapping and distinct intracellular sites for the replication of each of these agents probably exist. In addition, the increased rate of Coxsackie virus-infected cell death in which a majority of the cells remain intact, is in sharp contrast to the more complete degeneration resulting from poliovirus infection.

The effects on suspended cells of a mixed infection with $10^{6.0}$ TCID50 per ml of Coxsackie virus and $10^{5.0}$ TCID50 per ml of

poliovirus are described in Figure 3. It is apparent that there was a lag in cell killing with a small proportion of the cells retaining viability up to 24 hours after inoculation, followed by a significant reduction of intact cells. Those cells with increasing permeability followed a curve intermediate between that found for cells infected with either virus alone. Virtually all of the Coxsackie virus was adsorbed by 4½ hours after inoculation, but 30% of the poliovirus inoculum remained free as unadsorbed virus or virus which had been eluted from cells (Figure 4). The fact that the poliovirus could not be washed free of singly infected suspended cells at this same time after infection would suggest that in mixed infection there is only a reversible adsorption to the majority of susceptible cells. These data are in accord with reports on mode of attachment of poliovirus to cells (2, 8, 21). Furthermore, that Coxsackie virus is responsible for this blocking effect is indicated by the later uptake of poliovirus by presumably uninfected susceptible cells when a marked loss occurred in

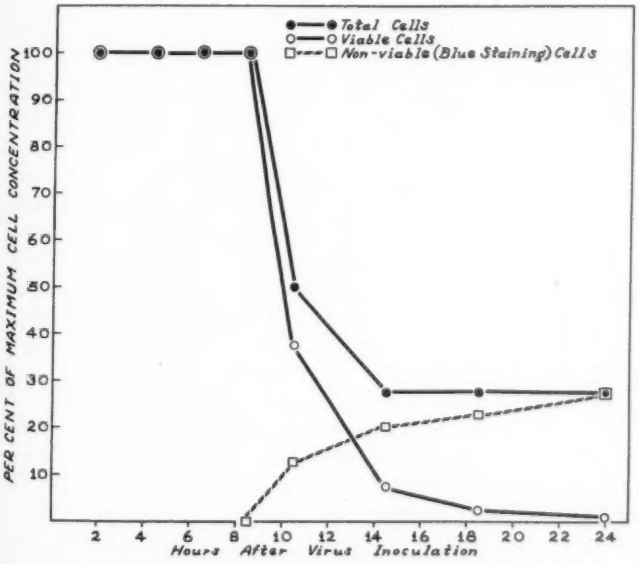


FIG. 3. Survival of suspended host cells (HeLa) mixedly infected with poliovirus and Coxsackie virus.

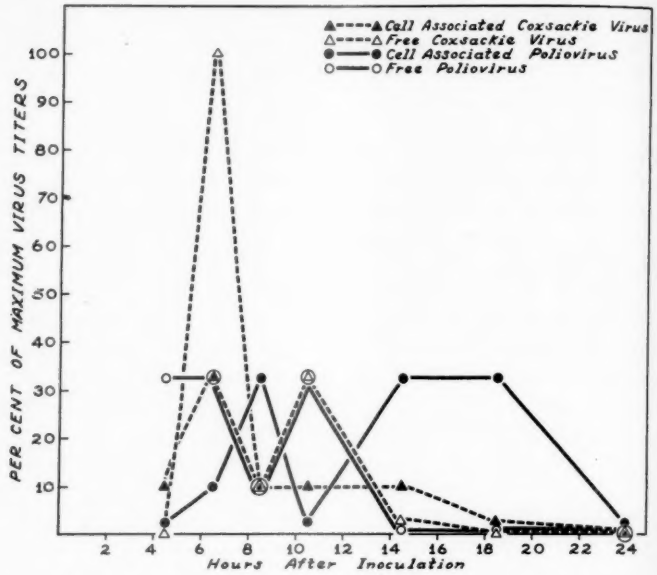


FIG. 4. Poliovirus and Coxsackie virus interactions in mixedly infected suspended cell (HeLa) culture.

both cell-associated and free Coxsackie virus fractions. The increase in free Coxsackie virus, in mixed infection, accompanied by a rise in the cell-associated virus titer (similar but not identical with that found in singly infected cells) would suggest a mutual exclusion by the poliovirus. Free poliovirus was usually reduced to 10% of the amount contained in the inoculum by 8½ hours after initiation of infection. This decline correlated directly with a rise in cell-associated virus indicating that cells still susceptible to poliovirus had been altered by contact with Coxsackie virus at some early stage of the poliovirus growth cycle. It is also conceivable that only small numbers of cells are initially infected with poliovirus resulting in the relatively low virus yield which may be further reduced by a Coxsackie virus-induced inhibition at the maturation and/or release phase of poliovirus growth. Such an inhibition could account for the delay in appearance of cell-associated poliovirus which theoretically ought to have been and was accompanied by a decrease in Cox-

sackie virus titer. This may also explain the high ratio of cell-associated to free poliovirus late in the course of the experimental mixed infection. A second peak of free Coxsackie virus production occurred concomitantly with a liberation of poliovirus. Since there was no simultaneous rise in the cell-associated Coxsackie virus fraction it may be assumed that the degenerative process precedent to the liberation of poliovirus caused this abnormal release of Coxsackie virus. When most of the remaining intact cells were no longer viable only low levels of cell-associated and free Coxsackie virus and free poliovirus could be detected, while 30% of the maximum poliovirus titer could be demonstrated in association with such cells. This evidence would thus lend substance to the assumption that Coxsackie virus may inhibit poliovirus release and that viable cells are required for maximum replication of both viruses.

Experiments identical to these with suspension of the S3 clonal line of HeLa cells were carried out to determine the reproducibility of these results. Except for the fact that virus titrations were carried out in bottle monolayers by a plaque technique similar to that described by Darnell and Sawyer (9), the conditions and methods employed corresponded to those with the original HeLa cell line in suspension. Antisera for neutralization of each agent in mixed yields were obtained from the ascitic fluid of mice injected i.p. with Ehrlich ascites carcinoma cells at the time of the last of a series of i.m. virus inoculations as previously described by Miya, *et al.* (42). The data from these experiments confirmed the results of the previous studies. Poliovirus titers, however, were lower under the same conditions of production employed with the original HeLa cell line and the cells showed longer survival after inoculation. These findings are in agreement with the more recent reports of Darnell and Sawyer (10) who suggested that a reduced efficiency in removal of poliovirus protein from RNA by the S3 cell line in the early stages of infection accounted for their greater resistance to this agent. Mixed infection of this cell line disclosed no greater percent inhibition of the poliovirus by Coxsackie virus than that observed in suspensions of the original HeLa cell cultures. These data would therefore support the hypothesis that inhibition occurs at the adsorptive stage of poliovirus infection since adsorption rates of poliovirus were found to be identical in both cell lines (10). The fact that Coxsackie virus

was replicated at the same rate and with corresponding virus titers in both cell lines indicates a specificity of cellular resistance which remains to be investigated.

Discussion

Reciprocal effects observed in mixed infections with Coxsackie virus and poliovirus in a variety of host systems have been described in terms of protection against or enhancement of cytopathogenesis, symptomatology and mortality, as well as on the basis of the proliferation of one or the other, or both viruses. Quantitation of viral activity has revealed exclusion, enhancement, and dual infection. In attempts to define basic mechanisms involved it appears that in order for the Coxsackie virus to interfere with poliovirus infection in mice it must be present in the same tissue, either through route of inoculation or by its specific ability to progress through the animal to mutual sites of predilection. The fact that ordinarily insusceptible adult mice seem to support the growth of certain Coxsackie viruses only on subsequent injection of poliovirus, together with the evidence that Coxsackie virus must reach certain levels in the central nervous system to affect a "blocking" of the poliomyelitic infection, substantiates the conclusions of Sulkin, *et al.* (56) that these interactions depend upon the pre-emption of susceptible cells. The lack of this "blocking" effect by Group A Coxsackie viruses might be attributed to the inability of the serotypes used to multiply in central nervous system tissue with or without an enhancing stimulus by subsequent poliovirus inoculation. Unfortunately, however, the variations in effects found by alteration of serotype, dosage, route of inoculation, and relative susceptibility of the animals by age selection give little information concerning the fundamental mechanisms involved in these complex interactions.

A comparison of the growth cycles of these agents in suspended cells suggests notable differences in both proliferative and cell degenerative activities which may account for some of the observed interactions in mixedly infected experimental animals and in cell monolayer cultures. Major differences seem to be related to the liberation of Coxsackie virus at the same time as it matures in infected cells in contrast to the delay that exists between the time of appearance of mature cell-associated poliovirus

and its release. A similar delayed release of poliovirus was observed by Howes (22) who suggested on the basis of experiments with isolated, infected single cells, that this apparent lag was due to a variation in time of appearance of first mature virus. Furthermore, poliovirus-infected cells showed increasing permeability and then lysis prior to the liberation of free virus while Coxsackie virus-infected cells released virus at approximately the same time as the occurrence of increased cell permeability and disintegration. In fact, the survival of mixedly infected suspended cells proved to be an extremely sensitive indicator of the protective effect observed in animals and in monolayer cultures of the same cells.

That little dual infection of individual suspended cells occurs is suggested by the delay in poliovirus uptake in mixedly infected populations, as well as by the over-all inhibition of poliovirus proliferation. The limited dual infection which results in abnormal release of Coxsackie virus simultaneously with poliovirus would imply that increased cell permeability may play a role in the mechanisms by which poliovirus enhances the proliferative activity of Coxsackie virus in mice. This may also be related to the additive effect noted with certain Coxsackie viruses and poliovirus in monkeys (7). The fact that no extraviral substance was found to mediate these viral interactions indicates that the Coxsackie virus particle itself is responsible for the observed effects.

Although adsorption rates of both agents are comparable in the original and the S3 HeLa cell lines both produced more infectious units of Coxsackie virus than poliovirus. This finding appears to be in keeping with the observed differences in the post-adsorptive phases of the growth cycles of each of these agents as related to their individual effects on cells and modes of free virus production. Furthermore, although the results of mixed infections of monolayers were not as marked as those of suspended cell cultures, several distinct functions of Coxsackie virus particles could be identified and related to previously described viral interactions. The diverse activities demonstrated by standard and inactivated Coxsackie virus remain, however, to be fully characterized. It seems clear that the mechanisms involved in these heterologous viral interactions, *in vivo*, may be explained on the basis of biological functions in host-viral relationships at the cellular level.

Summary and Conclusions

The pathogenic effects on mice of mixed infection with Coxsackie virus and poliovirus may be altered by variations in virus serotype, dosage, route of inoculation and age of the animals. Prior intracerebral inoculation of Coxsackie viruses (B-1 and B-4) afforded no protection to adult mice against intracerebral challenge with type 2 poliovirus, while intracerebral and intraperitoneal inoculation of Coxsackie virus (B-3) limited the progress of poliomyelitis in these animals. In the latter experiments proliferation of Coxsackie virus was observed only in those animals that had received subsequent poliovirus injections. No evidence for interference with poliovirus infections of adult mice was obtained when prior inoculation of different strains of Coxsackie virus (A-1, A-2, A-3 and 2 A-4) was employed. A Coxsackie virus (A-4), however, enhanced subsequent poliovirus infection by decreasing the incubation period. In addition, it was found that prior inoculation of immature mice with poliovirus resulted in a sparing effect on the early stages of infection with Coxsackie virus (A-4). These studies therefore provided further support for the assumption that pre-emption of certain cells by one agent is requisite for alterations in the progress of the other. A change in specific susceptibility of animals by age selection, or by use of agents with differing tissue tropisms caused anticipated variation in experimental results.

Studies of mixed infections of several tissue culture systems with active and inactivated agents suggested possible mechanisms involved in the *in vivo* experiments. Pretreatment of HeLa cell monolayer with active Coxsackie virus (B-3) increased the time for the appearance of poliovirus-induced cytopathogenesis while ultraviolet-inactivated Coxsackie virus decreased this interval. Further studies provided evidence that these effects were due to the activity of the Coxsackie virus particle *per se* rather than to a suspected extraviral component.

Suspended original HeLa cell cultures, as well as the S3 line, infected with each agent demonstrated striking variations in cycles of virus multiplication and cell degenerative activities. Such cell cultures exposed to both agents simultaneously exhibited a delay in cytopathogenesis in addition to reciprocal viral interactions. An inhibition of poliovirus adsorption and release occurred along with a late, abnormally high ratio of free to cell-

associated Coxsackie virus. The latter observation may have been due to a premature release of Coxsackie virus from doubly infected cells by the increased permeability and disintegration characteristic of poliovirus infection.

From these data it appears likely that the reciprocal effects observed in mixed infection with Coxsackie and poliovirus *in vivo*, may be explained on the basis of the results obtained from experiments in tissue culture systems.

REFERENCES

1. Adams, J. M., R. A. Boak, C. M. Carpenter, J. D. French, S. L. Klein, J. J. Pressman, and J. L. Smith: Prevention of paralytic poliomyelitis in tonsillectomized cynomolgus monkeys by human gamma globulin. *J. Lab. Clin. Med.*, 41: 142, 1953.
2. Bachtold, J. G., H. C. Bubel, and L. P. Gebbardt: The primary interaction of poliomyelitis virus with host cells of tissue culture origin. *Virology*, 4: 582, 1957.
3. Buckley, S. M.: Visualization of poliomyelitis virus by fluorescent antibody. *Arch. f. d. ges. Virusforsch.*, 4: 388, 1955.
4. Dalldorf, G.: The Coxsackie viruses. *Bull. N.Y. Acad. Med.*, 26: 329, 1950.
5. Dalldorf, G.: The sparing effect of Coxsackie virus infection on experimental poliomyelitis. *J. Exptl. Med.*, 94: 65, 1951.
6. Dalldorf, G., and E. Whitney: A further interference in experimental poliomyelitis. *Science*, 98: 477, 1943.
7. Dalldorf, G., and H. Wiegand: Poliomyelitis as a complex infection. *J. Exptl. Med.*, 108: 605, 1958.
8. Darnell, J. E., Jr.: Adsorption and maturation of poliovirus in singly and multiply infected HeLa cells. *J. Exptl. Med.*, 107: 633, 1958.
9. Darnell, J. E., Jr., and T. K. Sawyer: Variation in plaque forming ability among parental and clonal strains of HeLa cells. *Virology*, 8: 223, 1959.
10. Darnell, J. E., Jr., and T. K. Sawyer: The basis for variation in susceptibility to poliovirus in HeLa cells. *Virology*, 11: 665, 1960.
11. Domok, I.: Studies on the interaction between Coxsackie and poliomyelitis viruses. I. Simultaneous infection with B1 Coxsackie and Lansing poliomyelitis viruses in mice of different ages. *Acta. Microbiol. Acad. Sci. Hung.*, 4: 183, 1957.
12. Domok, I.: Studies on the interaction between Coxsackie and poliomyelitis viruses. II. Late resistance to poliomyelitis of young mice previously infected with Coxsackie B1 virus. *Acta Microbiol. Acad. Sci. Hung.*, 5: 111, 1958.
13. Domok, I.: Studies on the interaction between Coxsackie and poliomyelitis viruses. III. The course of resistance to poliomyelitis virus induced by Coxsackie B1 virus in young mice. *Acta Virologica*, 3: 222, 1959.
14. Dunnebacke, T. H.: Cytopathic changes associated with poliomyelitis infections in human amnion cells. *Virology*, 2: 811, 1956.
15. Girardi, A., H. McMichael, and W. Henle: The use of HeLa cells in suspension for the quantitative study of virus propagation. *Virology*, 2: 532, 1956.

16. Godman, G. C., H. Bunting, and J. L. Melnick: The histopathology of Coxsackie virus infection in mice. I. Morphologic observations with four different viral types. *Am. J. Pathol.*, 28: 223, 1952.

17. Habel, K., and C. P. Li: Intraspinal inoculation of mice in experimental poliomyelitis. *Proc. Soc. Exper. Biol. and Med.*, 76: 357, 1951.

18. Halperen, S., and S. E. Sulkin: Unpublished observations.

19. Henle, W.: Interference phenomena between animal viruses: A Review. *J. Immunol.*, 64: 203, 1950.

20. Hitchcock, G., and D. A. J. Tyrell: Some virus isolations from common colds. II. Virus interference in tissue cultures. *The Lancet*, 7118: 237, 1960.

21. Holland, J. J., and L. C. McLaren: The mammalian cell-virus relationship. II. Adsorption, reception, and eclipse of poliovirus by HeLa cells. *J. Exptl. Med.*, 109: 487, 1959.

22. Howes, D. W.: The growth cycle of poliovirus in cultured cells. II. Maturation and release of virus in suspended cell population. *Virology*, 9: 96, 1959.

23. Howes, D. W.: Growth cycles of poliovirus in cultured cells. III. The asychronous response of HeLa cells multiply infected with type 1 poliovirus. *Virology*, 9: 110, 1959.

24. Howes, D. W., and J. L. Melnick: The growth cycle of poliovirus in monkey kidney cells. I. Maturation and release of virus in monolayer cultures. *Virology*, 4: 97, 1957.

25. Howitt, B. F., and V. J. Nichols: Inoculation of cynomolgus monkeys with Coxsackie virus alone, combined, or with poliomyelitis virus. *J. Immunol.*, 68: 599, 1952.

26. Isaacs, A.: Viral interference. "Virus growth and Variation," *Ninth Symp. Soc. Gen. Microbiol.* p. 102, 1959.

27. Isaacs, A., and D. C. Burke: Viral interference and interferon. *Brit. Med. Bull.*, 15: 195, 1959.

28. Isaacs, A., and J. Lindenmann: Virus interference. I. The interferon. *Proc. Roy. Soc. B.* 147: 258, 1957.

29. Karber, G.: Beitrag zur kollektiven Behandlung pharmakologischer Reihennersuche. *Arch. f. exper. Path. u. Pharmakol.*, 162: 480, 1931.

30. Kilbourne, E. D., and J. S. Murphy: Gentic studies of influenza viruses. I. Viral morphology and growth capacity as exchangeable genetic traits. Rapid *in ovo* adaptation of early passage Asian strain isolates by combination with PR8. *J. Exptl. Med.*, 111: 387, 1960.

31. Le Bouvier, G. L.: Interference and cell protection by poliomyelitis virus in tissue culture. *Nature*, 174: 649, 1954.

32. Levaditi, C.: Association entre ultravirus: Poliomyelitis, (souche Lansing) et rage des rues. *Compt. rend. Soc. de Biol. Paris*, 137: 639, 1943.

33. Levaditi, C., and A. Vaisman: Relations entre les virus "Coxsackie" et poliomyeliete. *Ann. Inst. Pasteur.* 80: 678, 1951.

34. Lwoff, A., R. Dulbecco, M. Vogt, and M. Lwoff: Kinetics of release of poliomyelitis virus from single cells. *Virology*, 1: 128, 1955.

35. Lycke, E.: Interference between poliomyelitis virus and Coxsackie B or ECHO viruses. *Arch f. d. ges. Virusforsch.*, 8: 352, 1958.

36. Melnick, J. L.: Studies on the Coxsackie viruses: Properties, immunological aspects and distribution in nature. *Bull. N. Y. Acad. Med.*, 26: 342, 1950.

37. Melnick, J. L., and A. S. Kaplan: Dual antibody response to Coxsackie and poliomyelitis viruses in patients with paralytic poliomyelitis. *Proc. Soc. Exptl. Biol. Med.*, 74: 812, 1950.

38. Melnick, J. L., and A. S. Kaplan: Quantitative studies of the virus-host relationship in chimpanzees after inapparent infection with Coxsackie viruses. I. The virus carrier state and the development of neutralizing antibodies. *J. Exptl. Med.*, 97: 367, 1953.

39. Melnick, J. L., A. S. Kaplan, E. Zabin, G. Contreras, and N. Larkum: An epidemic of paralytic poliomyelitis characterized by dual infections with poliomyelitis and Coxsackie viruses. *J. Exptl. Med.*, 94: 471, 1951.

40. Melnick, J. L., N. Ledinko, A. S. Kaplan, and L. M. Kraft: Ohio strains of a virus pathogenic for infant mice (Coxsackie group). Simultaneous occurrence with poliomyelitis virus in patients with "summer grippe." *J. Exptl. Med.* 91: 185, 1950.

41. Melnick, J. L., E. W. Shaw, and E. C. Curnen: A virus isolated from patients diagnosed as non-paralytic poliomyelitis or aseptic meningitis. *Proc. Soc. Expt. Biol. Med.*, 71: 344, 1949.

42. Miya, F., G. A. Hill, and S. Marcus: Serologic analysis of Ehrlich tumor-cell induced ascites fluid. *Proc. Soc. Exptl. Biol. Med.* 102: 382, 1959.

43. Morgan, C., C. Howe, and H. M. Rose: Intracellular crystals of Coxsackie virus viewed in the electron microscope. *Virology*, 9: 145, 1959.

44. Okuno, Y., A. Aoyama, N. Tsujimoto, K. Kusumoto, T. Hotta, T. Sugai, S. Ishii, S. Ebara, and S. Fukae: Diagnosis of virus propagation by the interference method. *Med. J. Osaka U.* 7: 257, 1956.

45. Proc. First International Conference on Live Poliovirus Vaccines, 1959, Washington, D. C., *Scientific Publication No. 44*, Pan American Sanitary Bureau.

46. Reed, L. J., and H. Muench: A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.*, 27: 492, 1938.

47. Reissig, M., D. W. Howes, and J. L. Melnick: Sequence of morphological changes in epithelial cell cultures infected with poliovirus. *J. Exptl. Med.* 104: 289, 1956.

48. Ruska, H., D. C. Stuart, Jr., and J. Winsser: Electron microscopic visualization of intranuclear virus-like bodies in epiethelial cells infected with poliomyelitis virus. *Arch. f. d. ges. Virusforsch.*, 6: 379, 1956.

49. Sacerdote de Lustig, E., and S. Brieux. Interference between Coxsackie and poliomyelitis viruses in HeLa cell cultures. *Compt. rend. Soc. de Biol.* Paris 150: 2017, 1956.

50. Salk, J. E., L. J. Lewis, J. S. Youngner, and B. L. Bennett: The use of adjuvants to facilitate studies on the immunologic classification of poliomyelitis viruses. *Am. J. Hyg.*, 54: 157, 1951.

51. Schlesinger, R. W.: Interference between animal viruses. in Burnet, F. M., and W. M. Stanley, *The Viruses*, New York, Academic Press 3: 157, 1959.

52. Stanley, N. F.: Attempts to demonstrate interference between Coxsackie and poliomyelitis viruses in mice and monkeys. *Proc. Soc. Exptl. Biol. Med.*, 81: 430, 1952.

53. Sulkin, S. E.: The Coxsackie viruses. Properties, immunological aspects and role in human disease. *Southern Med. J.*, 45: 516, 1952.

54. Sulkin, S. E.: Unpublished observations.

55. Sulkin, S. E., H. C. Wallis, and P. Donaldson: Differentiation of Coxsackie viruses by altering susceptibility of mice with cortisone. *J. Infect. Disease.* 91: 290, 1952.

56. Sulkin, S. E., H. C. Wallis, and T. P. Murphy, Jr.: Mixed infections with Coxsackie and Lansing poliomyelitis viruses. *Proc. Soc. Exptl. Biol. Med.*, 84: 184, 1953.

57. Taylor, H. M., and D. H. Sprunt: Increased resistance to viral infection as a result of increased fluid in tissues. *J. Exptl. Med.*, 78: 91, 1943.

58. Vilches, A., and G. K. Hirst: Interference between neurotropic and other unrelated viruses. *J. Immunol.*, 57: 125, 1947.

CYTOLOGICAL STUDIES OF TUMORS, XXXVI. ALTERATIONS OF CELL POPULATIONS IN THE MTK-SARCOMA II AFTER SOME EXPERIMENTAL PROCEDURES*

YOSHIKAZU MATANO AND SAJIRO MAKINO

Several items of evidence have been collected during the last several years from cytological analysis of tumor cells in the rat and mouse to show that the constancy of the stemline, while very pronounced, is not necessarily permanent, and that transplantable ascites tumors during serial transfers have undergone numerical and structural changes of chromosomes (Levan 1956, Makino 1957, Tonomura and Sasaki 1957, Makino and Sasaki 1958, Yosida 1959). The present communication presents examples to demonstrate that tumor cell population *in vivo* can be altered experimentally.

The MTK-sarcoma II is one of the transplantable ascites tumors of the rat produced by means of a long-term administration of azo dyes (Tanaka and Kano 1951). In early transfer generations, the MTK-sarcoma II was characterized by stem cells, the subdiploid chromosome complement being 40 or thereabouts in number, with one V-shaped element of outstandingly large size as a marker chromosome, by the presence of which the present tumor was clearly distinguishable from the normal somatic tissues, as well as from some other tumors of like nature (Makino and Kano 1953, Tonomura 1954). Later Tonomura and Sasaki (1957) found that numerical and morphological changes of the chromosomes had taken place in this tumor; the stem cells contained two V-shaped marker-chromosomes with a modal chromosome number of 39. Very recently a further shift was found to occur in the stemline chromosome pattern of this tumor. The present paper describes the most recent stemline chromosome-pattern of the MTK-sarcoma II, together with evidence demonstrating that some experimental procedures could induce alterations in frequency of cell populations.

* Contribution No. 509 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan. Aided by a grant from Damon Runyon Memorial Fund for Cancer Research to S. Makino (DRG-563).

Materials and Methods

The rats used in the following experiments with the MTK-sarcoma II were purely inbred Wistar albinos, weighing about 100 grams.

The chromosomes were studies under the following four experimental schemes:

1. Tumor cells, obtained from the 493rd to 583rd transfer generations, provided the control material.
2. The chromosomes were observed in the tumor after the treatment of triethylene melamine (TEM, Harold Heifetz Chemical Laboratories): a suspension prepared with 1 mg of TEM and 100 cc of a physiological saline solution was used for intraperitoneal injection. A single injection was given to each tumor-bearing rat on the third day after transplantation at dose levels of 0.05 or 0.1 mg per 1 kg of body weight.
3. A piece of the tumor nodule, which was developed on the omemtum of a tumor-bearing rat, was removed; after being washed with Rinaldini's solution, containing 0.15 per cent trypsin, it was minced with small scissors and intraperitoneally transplanted into a new rat. The tumor cells developed well in the peritoneal cavity of the new host, forming a new cell-population in ascites form. The chromosomes were studied in tumor cells thus developed on the basis of the samples derived on the third day after transplantation.
4. The chromosomes were investigated in a single cell-clone isolated from the stock tumor (Tonomura and Matano, unpublished).

For observation of the chromosomes a water pretreatment squash method (Makino 1957) was adopted advantageously.

Results

I. *The chromosomes of the stock tumor.*

In every sample derived from the most recent tumor-bearing rats, it was found that over 95 per cent of tumor cells at metaphase was near-diploid, with the chromosome number varying from 37 to 43, the most frequent number being 39 (Table 1). The cells contained 1 to 4 prominently large metacentric chromosomes provided with median or submedian centromeres: they are clearly distinguishable from others. In the following descriptions, those particular chromosomes are designated as m or sm chromosomes, the former referring to median chromosomes and the latter to submedian ones. In Table 2 is given the frequency distribution of various cell-types in serial transfers ranging from the 493rd to 583rd generation with special reference to the m and sm chromosomes. It was shown that cells, having 2m chromosomes, together with sm chromosomes, and those having 2m chromosomes only, were very prominent among others, both types of cells having 39 as their total chromosome number. In the follow-

TABLE 1

Distribution of chromosome-numbers in serial transfers of the MTK-sarcoma II showing a mode at 39

Transfer generation	Chromosome number							Total no. of cells observed
	37	38	39	40	41	42	43	
493rd–503rd	..	4 (8.3%)	31 (64.6%)	10 (20.8%)	3 (6.3%)	..	..	48 (100.0%)
532nd–534th	..	6 (6.0%)	81 (81.0%)	13 (13.0%)	..	..	..	100 (100.0%)
570th–571st	1 (2.0%)	1 (2.0%)	32 (64.0%)	11 (22.0%)	3 (6.0%)	1 (2.0%)	1 (2.0%)	50 (100.0%)
580th–583rd	1 (1.6%)	6 (9.4%)	38 (59.4%)	15 (23.4%)	2 (3.0%)	1 (1.6%)	1 (1.6%)	64 (100.0%)
Total number of cells observed	2 (0.8%)	17 (6.5%)	182 (69.3%)	49 (18.7%)	8 (3.1%)	2 (0.8%)	2 (0.8%)	262 (100.0%)

ing descriptions, the former type of cells are referred to as 2m-1 sm type cells, and the latter as 2m type cells (Figs. 1 and 2). Ideogram analysis was made in three adequate metaphasic cells in each of the two cell-lines, following the system of Tjio and Levan (1956) with some modifications of arm index: M chromosome carries a subterminal one, and T chromosome is characterized by a centromere localized in its terminal region, though the short arm is not easily detectable. The numbers of M, S, and T chromosomes in the two types of cells were found as follows:

	Number of		
Cell-type	M	S	T
2m-1 sm	13	13	13
2m	12	13	14

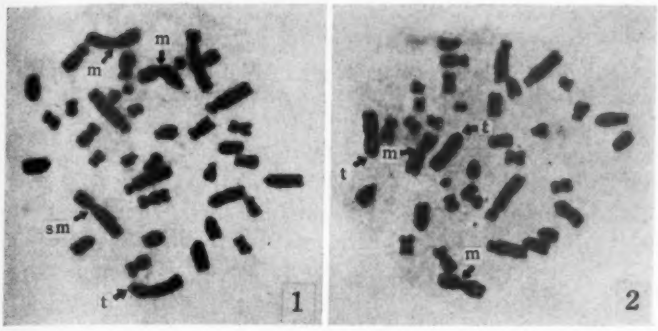
A morphological comparison of the chromosomes was made between the two cell-lines (Figs. 3–8). It was shown that they were different in number of M chromosomes: 2m-1 sm type cells contained 13 M chromosome consisting of 2m-1 sm, and 10 small M elements, while 2m type cells included 12 M chromosomes, two of which were m chromosomes: the remaining ten were small M elements, quite identical with the corresponding ones occurring in the former type cells. They differed again in number of T chromosomes: The T chromosome constitution of 2m type cells was 14 in number, consisting of two outstandingly large and nearly equal elements, nine medium-sized and three very small ones, whereas 2m-1 sm type cells contained 13 T ele-

TABLE 2

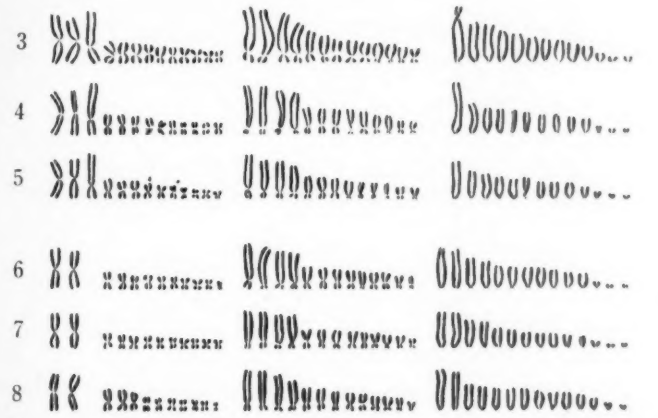
Frequency distribution of various cell-types during serial transfers of the MTK-sarcoma II with special regard to "m-" and "sm-" chromosomes

Chrom. no.	Cell type	Transfer generation				Total no. of cells observed
		493rd–503rd	532nd–534th	570th–571st	580th–583rd	
37	2m-1sm	.....	.....	.....	1(1.6%)	1(0.4%)
	1m-1sm	.....	.....	1(2.0%)	.....	1(0.4%)
	3m-1sm	.....	.....	.....	1(1.6%)	1(0.4%)
38	2m-2sm	2(4.2%)	.....	.....	.....	2(0.8%)
	2m-1sm	2(4.2%)	5(5.0%)	1(2.0%)	4(6.3%)	12(4.6%)
	1m-1sm	.....	1(1.0%)	.....	1(1.6%)	2(0.8%)
	2m-2sm	2(4.2%)	.....	.....	.....	2(0.8%)
	3m	.....	1(1.0%)	.....	.....	1(0.4%)
	2m-1sm	22(45.8%)	55(55.0%)	21(42.0%)	28(43.8%)	126(48.3%)
39	1m-2sm	2(4.2%)	5(5.0%)	1(2.0%)	1(1.6%)	9(3.4%)
	2m	5(10.4%)	13(13.0%)	6(12.0%)	5(7.8%)	29(11.1%)
	1m-1sm	.....	3(3.0%)	3(6.0%)	3(4.7%)	9(3.4%)
	1m	.....	4(4.0%)	1(2.0%)	1(1.6%)	6(2.3%)
	2m-2sm	1(2.1%)	.....	.....	.....	1(0.4%)
	3m	.....	2(2.0%)	.....	.....	2(0.8%)
	2m-1sm	7(14.6%)	6(6.0%)	5(10.0%)	10(15.6%)	28(10.7%)
40	1m-2sm	2(4.2%)	.....	1(2.0%)	1(1.6%)	4(1.5%)
	2m	.....	2(2.0%)	3(6.0%)	4(6.3%)	9(3.4%)
	1m-1sm	.....	2(2.0%)	1(2.0%)	.....	3(1.2%)
	1m	.....	1(1.0%)	1(2.0%)	.....	2(0.8%)
	2m-1sm	3(6.3%)	.....	1(2.0%)	.....	4(1.5%)
	3m	.....	.....	.....	1(1.6%)	1(0.4%)
41	1m-2sm	.....	.....	1(2.0%)	.....	1(0.4%
	2m	.....	.....	1(2.0%)	.....	1(0.4%)
	1m	.....	.....	.....	1(1.6%)	1(0.4%)
42	2m-2sm	.....	.....	1(2.0%)	.....	1(0.4%)
	2m	.....	.....	.....	1(1.6%)	1(0.4%)
43	2m-1sm	.....	.....	1(2.0%)	.....	1(0.4%)
	2m	.....	.....	.....	1(1.6%)	1(0.4%)
Total number of cells observed		48(100.0%)	100(100.0%)	(50(100.0%	64(100.0%)	262(100.0%)

ments, one of which was conspicuously large-sized, the remaining twelve similar to those found in the former. The two cell-lines were, however, nearly identical in regard to S chromosomes



FIGS. 1–2. Photomicrographs of chromosomes of two cell-types of the MTK-sarcoma II, 39 chromosomes in each, × 1500. m: the large chromosomes with a median centromere, sm: the large chromosome with a submedian centromere, t: the largest T chromosome.



FIGS. 3–8. Ideogram analysis in two cell-types of the stock MTK-sarcoma II. 3–5, 2m–1sm type-cells. 6–8, 2m type-cells.

since there were four considerably large and nine small S chromosomes showing a gradual order of size.

In the light of the above findings, the conclusion was derived that the two cell-lines were different in their chromosome constitution since 2m type cells contained two extremely large T chromosomes but no sm chromosomes, while 2m-1 sm type cells possessed one corresponding large T chromosome and 1 sm ele-

ment similar in length to the former element. There is no evidence to speculate that the sm chromosomes occurring in 2m-1 sm type cells might have been derived as a result of pericentric inversion or centromere shift in one of the extremely large T chromosomes occurring in 2m type cells (for detail, see Matano 1960). It appears therefore that 2m-1 sm type cells might be descendants from 2m type cells.

It should be noted that the ideogram of the 2m type cells is identical to that of 2V type cells which were established as stem-cells of an original strain of this sarcoma by Tonomura and Sasaki (1957).

In serial transfers of this tumor, these two cell-lines have served as stemlines contributing to the neoplastic growth in Wistar rats, and have maintained their chromosome patterns without shift. As shown in Table 2 the two types of cells have maintained their occurrence in tumor ascites, with a rather constant frequency of about 50 per cent for the 2m-1 sm type cells and about ten per cent for the 2m type cells.

III. *Alterations in frequency of two cell-lines after some experimental procedures.*

1) *After TEM-treatments*: The behavior of the two cell-lines was studied under the influence of TEM. The application of TEM to the tumor-bearing rats induced a remarkable depression of mitotic rate of tumor cells. Subsequently mitotic activity increased strikingly with abnormal mitoses: about 24 hours after injection, almost all cells showed abnormal mitoses indicating remarkable structural changes of the chromosomes. By 40 hours after injection, the tumor cells gradually recovered from the chemical damage, showing normal mitotic behavior. By 72 hours after treatment, a complete regrowth of the tumor was attained.

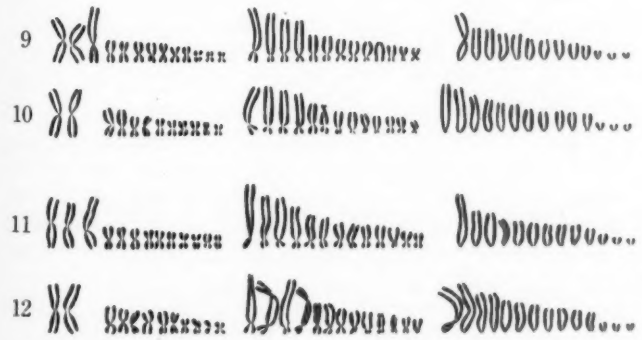
The chromosomal features of tumor cells under the effect of TEM were investigated just before and five to eight days after its injection. The behavior of the two cell-lines following TEM treatments are summarized in Table 3. It was shown that under the influence of TEM, 2m-1 sm type cells significantly decreased in number after treatment, whereas 2m type cells showed a tendency to increase in number. This was particularly evident in experiment II in which the number of the 2m type cells increased remarkably, predominating over that of the 2m-1 sm type ones.

TABLE 3

Behavior of two cell-lines of the MTK-sarcoma II after a single injection of TEM

Host condition	Cell type	Frequency of two cell-types based on near-diploid cells		
		Exp. I (0.05 mg/kg)	Exp. II (0.1 mg/kg)	Exp. III (0.1 mg/kg)
Before injection	2m-1sm	45.8%	55.0%	42.0%
	2m	10.4%	13.0%	12.0%
Complete recovery, 5 to 8 days after injection	2m-1sm	24.0%	30.0%	26.0%
	2m	12.0%	32.0%	16.0%
9th transfer generation after injection	2m-1sm	...	...	37.2%
	2m	...	...	11.9%

Ideogram analyses were undertaken with samples derived from experiments II and III before and after TEM-injection on the basis of three metaphasic cells in each cell-line. These showed that the chromosome constitution was not altered by the treatment, the stem-cells persisting in their characteristic chromosome constitution (Figs. 9 and 10). The tumor cells were then sampled on the sixth day after TEM treatment, and transplanted into untreated Wistar rats successively for nine generations. In the ninth generation, the chromosomes were examined (Exp. III in Table 3). The results showed that both frequency and ideo-



FIGS. 9–12. Ideogram analysis in two cell-types after experimental conditions. 9–10, after TEM-treatment. 11–12, after transplantation of a metastatic tumor nodule.

gram of the two stemline cells were similar to those of controls. It appears then that the change in frequency of the two types of cells, occurring after TEM treatments, is a result of response of tumor cells to the chemical treatment.

2) *After transplantation of a metastatic tumor nodule*: The chromosomes were investigated on the third day after peritoneal inoculation of the tumor nodule metastatic on the omentum. The results are shown in Table 4. The 2m-1 sm type cells showed a

TABLE 4

Behavior of two cell-lines of MTK-sarcoma II after the transplantation of a metastatic tumor nodule

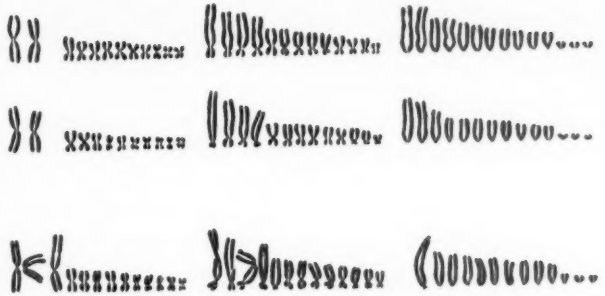
Host condition	Frequency of two cell-types based on near-diploid cells	
	2m-1sm type cells	2m type cells
Control (580th–583rd generations)	43.8%	7.8%
After peritoneal inocul. of tumor nodule	66.6%	3.9%
11th transfer generation after transplantation	40.0%	8.0%

striking increase to 66.6 per cent of dividing cells with a corresponding decrease of 2m type cells to merely 3.9 per cent. This feature is in inverse relation to the results obtained in the TEM experiments.

The tumor appeared in an ascites form after having been transferred intraperitoneally in Wistar rats for 11 generations. At the 11th generation the frequency of the stemline cells was observed. The results indicated a frequency very similar to that of controls with stemline ideograms unaltered (Figs. 11 and 12).

3) *In a single cell-clone*: Single cell inoculation experiments were performed with the stock tumor by Tonomura and Matano (unpublished), and a clone of 2m type cells was established. Ideogram analysis has shown that this clone was characterized by stem-cells having an ideogram identical with that of the 2m type cells of the stock tumor (Figs. 13 and 14).

The chromosome analysis was carried out in this single cell-clone with about 600 metaphasic cells derived from the first, second, third, fourth, fifth, sixth, seventh, eighth, ninth, tenth, twentieth and thirtieth transfer generations (about 50 cells in



FIGS. 13–15. Chromosomes of a single cell clone. 13–14, ideograms of the stem cells of the single cell clone. 15, ideogram analysis in an exceptional cell found in the single cell clone (2m–1sm type).

each generation). One exceptional cell was found in a sample from the 20th generation: it was characterized by two m chromosomes and one sm chromosome, 39 being the total number of chromosomes. A detailed ideogram analysis of this exceptional cell made it clear that it was similar in chromosomal constitution to 2m-1 sm type cells (see Matano 1960).

Discussion

The present observations have shown that the MTK sarcoma II, studied by the authors, is characterized by two distinct cell-lines different in their chromosomal constitutions, one consisting of 2m type cells and the other of 2m-1 sm type cells. The stock tumor was found to contain 2m-1 sm type cells at a rate of about 50 per cent of dividing cells, forming a superior line, together with 2m type cells at about ten per cent as an inferior line, predominating over 2m-1 sm type cells (Table 3). Different responses of the two cell-lines to chemicals applied are probably due to different drug resistance existing between the two cell types. Hauschka (1958) reported that drug sensitive tumors and their resistant sublines were different in their chromosome number and structure. In an ascites tumor, transformed from a metastatic solid tumor on the omentum, the 2m-1 sm type cells formed a superior line increasing markedly in frequency with a concomitant decrease of 2m type cells (Table 4). A single cell-clone isolated from the stock tumor was found to contain a predomi-

nant line consisting of 2m type cells. The ideogram analyses have revealed that the chromosome patterns of the 2m, as well as 2m-1 sm type cells, show at least morphologically no alteration from those found in the stock tumor before and after the TEM treatments, after transplantation of a metastatic tumor, and in a clonal line. Further it was found that the 2m type cells corresponded chromosomally to the 2V type cells reported by Tonomura and Sasaki (1957) in a strain of the MTK sarcoma II. The evidence presented here furnishes examples which demonstrate that tumor cell populations *in vivo* can be altered experimentally. Probably, experimental shocks may alter physiological conditions of the tumor-bearing rats which may lead to the change of growth pattern of the two cell-lines, apparently as a result of adaptation of cells to new conditions. Similar data, involving the adaptation of tumor cells to the physiological conditions of the host, have been presented in a human gastric carcinoma: two distinct cell-lines of this tumor, having different chromosome constitutions, showed a striking change in frequency value after radiation treatments of the patient (Makino *et al.* 1959).

Recently Hsu and Kellogg (1960) published a paper on an experimental alteration of cell populations *in vitro* working with strain L-P59, a subline of strain L clone 929 of the mouse. They showed that cell populations *in vitro* could be altered by means of long-term colchicine treatments. Their study suggests a method by which metabolically inferior mutants can be selected from cell populations *in vitro*. The present findings, derived from *in vivo* specimens from a transplantable ascites tumor of the rat, apparently approximate those of Hsu and Kellogg (1960) with *in vitro* specimens based on the mouse. A similar experiment was undertaken by Sasaki (1960), and one subline was selected through chemical treatments from a mixed population produced by a double inoculation of two different sublines of Yoshida sarcoma. It is interesting to see that there may be a remarkable similarity between the population structures of transplantable tumors and cell strains *in vitro*, though the environmental conditions *in vivo* are obviously much more complicated than those *in vitro*, since in the former the cell population is always controlled by physiological conditions of the host animal.

On the basis of their findings Hsu and Kellogg (1960) emphasized that "stemline" is a relative term depending upon the conditions of the environment, and that in a cell population there may be several stemlines, each being suitable for one type of an environment, and an equilibrium may be reached according to the environment provided for them. The present study seems to furnish some examples in favor of the above view.

Summary

The present investigations have revealed that the MTK-sarcoma II, a transplantable rat ascites tumor, is characterized by the occurrence of two distinct cell-lines, different in their chromosome constitution and frequency value. Evidence was presented to demonstrate that alterations in frequency of the two cell-lines could be induced experimentally.

The authors wish to thank Drs. Eiji Momma, Tatsuya Tanaka, Akira Tonomura and Motomichi Sasaki for their valuable criticisms. The authors are also indebted to Dr. T. C. Hsu for assistance in preparation of this manuscript.

REFERENCES

Hauschka, T. S.: Correlation of chromosomal and physiologic changes in tumors. *J. Cell. Comp. Physiol. 52, Suppl.*, 1: 197–267.

Hsu, T. C., and D. S. Kellogg: Mammalian chromosomes *in vitro*. XII. Experimental evolution of cell populations. *J. Nat. Cancer Inst.*, 24: 1057–1093, 1960.

Levan, A.: Chromosomes in cancer tissue. *Ann. N. Y. Acad. Sci.*, 63: 774–789, 1956.

Makino, S.: The chromosome cytology of th ascites tumors of rats, with special reference to the concept of the stemline cell. *Intern. Rev. Cytol.* 6: 25–84, 1957.

Makino, S., and K. Kanô: Cytological studies of tumors, IX. Characteristic chromosome individuality in tumor strain-cells in ascites tumors of rats. *J. Nat. Cancer Inst.* 13: 1213–1235, 1953.

Makino, S., and M. Sasaki: Cytological studies of tumors, XXI. A comparative ideogram study of the Yoshida sarcoma and its subline derivatives. *J. Nat. Cancer Inst.*, 20: 465–487, 1958.

Makino, S., T. Ishihara, and A. Tonomura: Cytological studies of tumors, XXVII. The chromosomes of thirty human tumors. *Zeits. Krebsf.*, 63: 184–208, 1959.

Matano, Y.: The rise of a new subline in the MTK-sarcoma II through the change of stemline chromosomes. *Jap. J. Genet.*, 35: 329–336, 1960.

Sasaki, M.: Cytological studies of tumors, XXVIII. Some experimental studies in two sublines of the Yoshida sarcoma, with special consideration of the deviation of sublines. *Cytologia*, 25: 69–85, 1960.

Tanaka, T., and K. Kanô: Cytological studies on cancer, IV. General characters of the MTK-sarcomas, new ascites tumors of rats produced by the administration of azo dye. *J. Fac. Sci. Hokkaido Univ. Ser. VI, Zool.*, 10: 289–301, 1951.

Tjio, T. H., and A. Levan: Comparative ideogram analysis of the rat and the Yoshida rat sarcoma. *Hereditas*, 42: 218–234, 1956.

Tonomura, A.: Cytological studies of tumors, XVI. Cytological differences of MTK-sarcoma II and Takeda sarcoma, with preliminary experiments on double inoculation with the two tumors. *J. Fac. Sci. Hokkaido Univ. Ser. VI, Zool.*, 12: 158–168, 1954.

Tonomura, A., and M. Sasaki: Cytological studies of tumors, XIX. A chromosome survey of the MTK-sarcoma II and III after several years of serial transfers. *J. Fac. Sci. Hokkaido Univ. Ser. VI, Zool.*, 13: 332–337, 1957.

Yosida, T. H.: Chromosomal alteration and development of tumors, I. A new subline of Yoshida sarcoma developed by chromosomal mutation. *Zeits. Krebsf.* 63: 209–214, 1959.

RAPID PROGRESSIVE COMA IN UNSUSPECTED INTRACRANIAL MENINGIOMAS*

Report of 4 Cases

ANSELMO PINEDA† AND JOHN E. COE

One of the usual features of intracranial meningiomas as a group is the long history of neurological signs and symptoms preceding the initial examination by a physician. These may be evaluated without unnecessary haste and the patient may be completely investigated before proceeding with elective surgery. (2, 5, 7, 9) However, some patients with meningioma have rapidly developing symptoms and signs which progress quickly to a critical state. Although this fact is recognized by most experienced neurosurgeons, a review of pertinent literature for the past 30 years did not show this to have been documented by papers dealing specifically with this problem.

In Cushing and Eisenhardt's (3) series published in 1938, 284 cases of intracranial meningioma were reported. Of these 53, roughly 20%, had the history of a tumor of only one year duration. Two cases had, respectively, a 3 week and 4 week history, while the balance had symptoms of 1 to 20 years duration.

In 1955 Hoessly and Olivecrona (8) reported 280 cases of parasagittal meningioma, 172 of which had a duration of history of 1 year or more. One hundred cases had over a two year history but 56 cases were reported to have less than a 6 month history and 17 cases, or approximately 6%, had less than a 3 month history.

Thus, although the great majority of patients with meningioma have a long history of slowly progressive neurological symptoms and signs (4, 14) an occasional individual is unconscious when first seen or has a brief history of convulsions, motor impairment, or headaches. These patients are usually

* From the Division of Neurological Surgery, Department of Surgery, The University of Texas School of Medicine, Galveston. Presented at the meeting of the Southern Neurosurgical Society, San Antonio, February 4, 1961. Submitted for publication April 15, 1961.

† Present address: Northwestern University Medical School, Chicago 11, Illinois.

considered to have either a cerebro-vascular accident or an intracranial glioma. Moore (13), in his report of ten cases of clinically unrecognized intracranial meningioma, described five who developed a sudden apoplectiform onset and were admitted with diagnosis of cerebral thrombosis or hemorrhage.

Four cases of clinically unsuspected meningioma with rapidly progressive course and unusually brief histories are presented here.

Cases

I. A 53 year old white female arrived in the emergency room in a comatose state. Apparently on the night before admission she retired early complaining of a very severe headache and was found comatose the next morning. On examination her blood pressure was 120/60; pulse 64; temperature 101.4° F. There was no papilledema, a questionable left Babinski, and no stiff neck. It was thought that the patient had suffered a cerebrovascular accident or had a possible glioma. A carotid arteriogram revealed a right parasagittal frontal lesion (Figure 1). "Tumor stain" in the early arterial phase and filling of the tumor from the external circulation was noted. These radiographic changes were suggestive of a meningioma. On further inquiry into the history, the husband revealed that the patient during the previous three years had complained quite vaguely of an occasional generalized headache which was relieved by aspirin, and at times she had complained of temporary weakness of her left arm. A craniotomy was performed and the meningioma was removed *in toto*. Postoperatively the patient slowly improved and was discharged, but

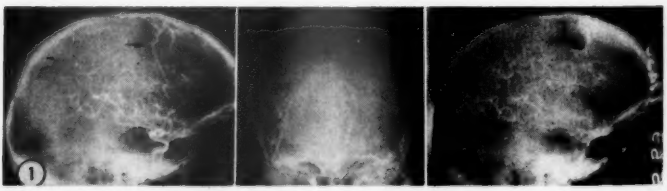


FIG. 1. Case 1. Parasagittal Meningioma. (a) carotid arteriography showing filling of abnormal vessels corresponding to the tumoral area, demonstrated by the arrow. Terminal branches of the middle cerebral artery being displaced posteriorly, (b) AP view with the shifting of the anterior cerebral toward the left side, (c) early filling to the parasagittal tumor from the external carotid artery branches. See staining as in (a).

we learned later that she died at home of unspecified cause six weeks after discharge.

II. The second patient was a 43 year old colored female who, during only the past two months before admission, had complained of questionable lethargy and weakness of the right arm and leg. Prior to this, she was in apparently good health. Shortly before admission she began to have generalized convulsions and projectile vomiting. Examination revealed a semi-comatose patient with blood pressure of 120/80 and a pulse of 80. Proptosis of the left eye and bilateral papilledema were observed. Routine skull x-rays were negative. A left carotid arteriogram revealed a "tumor stain" deep in the left frontal lobe with a shift of the anterior cerebral arteries to the right (Figure 2). The pre-opera-

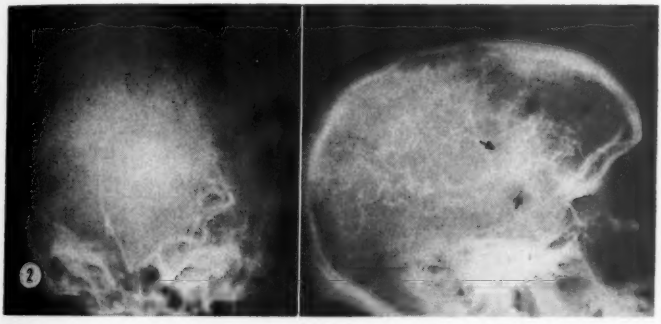


FIG. 2. Case 2. Sphenoidal Ridge Meningioma. (a) marked shifting of the anterior cerebral artery toward the right side, (b) "tumor stain" at the level of the lower frontal region. See collateral circulation from external circulation at the prefrontal area.

tive diagnosis was brain tumor, left frontal, probably a rapidly growing glioma; but at surgery a left sphenoid ridge meningioma was identified and removed. The patient was discharged, ambulatory but with greatly diminished vision.

III. The third patient, a 70 year old white female, presented a history of progressive unsteadiness of her gait for approximately six months. She had become quite confused and unable to co-operate. She had been bedridden for several months and her clinical diagnosis was cerebral arteriosclerosis. Although she was able to talk and respond well during all this time, she had become lethargic, began to vomit and became unconscious on the day of

admission. Physical examination revealed no particular abnormalities at the time of admission. Her blood pressure was 160/100; she did not have papilledema. Later on, bilateral Babinski responses were observed. A lumbar puncture done earlier that day revealed protein over 200 mgm% with xanthochromic fluid. Her neck was very stiff. Bilateral carotid arteriogram revealed displacement of the anterior cerebral artery to the left and a "tumor stain" just above the tentorium on the right with early filling of the area of the stain from the external circulation (Figure 3). She was operated the same day and

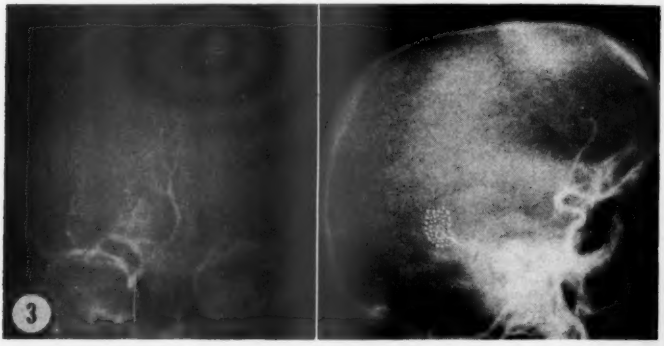


FIG. 3 Case 3. Meningioma arising from the tentorium. (a) shifting of the anterior cerebral artery not marked, (b) abnormal "staining" at the level of the tentorium and occipital region. Retouched for better demonstration.

following amputation of the occipital lobe, a meningioma arising from the tentorium on the right was identified and removed. Following two stormy post-operative days the patient gradually improved. On discharge she was ambulatory and had no complaints.

IV. Although patient number four did not present herself at the hospital with acute symptoms, she is of interest because of her rapidly worsening condition while in the hospital. She was a 57 year old white female who gave a history of gradually increasing generalized headaches of about one year duration. Six months before admission she noted a gradual increase in weakness of the right arm and leg. For two months she had noted failing vision and a decreasing sense of smell. General physical

examination was unrevealing except that it was thought by some examiners that the left common carotid artery was less palpable than the right. Blood pressure, 120/80; pulse 80. Neurological examination showed weakness of the entire right facial musculature, right arm and right leg. There was no papilledema. The patient was alert and co-operative. Visual fields showed a left scotoma. Three days after admission she became suddenly drowsy and lethargic. Her blood pressure dropped to 90/60. Carotid arteriography revealed a huge bifrontal lesion, more on the left (Figure 4). There was a "tumor stain" with early filling of the tumor area from the external circulation. A bifrontal craniotomy performed revealed a large meningioma attached to the left olfactory groove. The tumor was entirely removed and the patient discharged greatly improved with relatively little residual defect.

Discussion

The four cases presented here illustrate that intracranial meningiomas may be characterized by a very rapid development

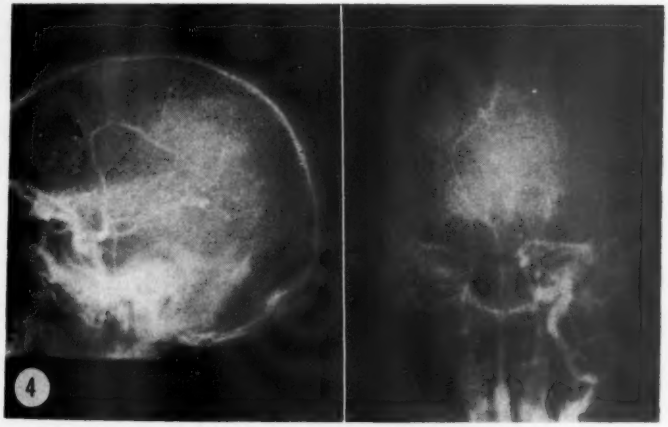


FIG. 4. Case 4. Meningioma of the Olfactory Groove. (a) left carotid arteriography demonstrating early circulation across the floor of the olfactory groove area and orbital roof. Marked displacement of the anterior cerebral artery upward and posteriorly, (b) AP view with pronounced shift of the anterior cerebral artery toward the right side, and the middle cerebral artery outwardly.

of acute symptoms of such a nature that the clinical picture may be easily confused with that of rapidly growing gliomas and even of acute vascular lesions. Cerebral arteriography differentiates these cases from vascular lesions and suggests the type of tumor present.

In clinical experience, loss of consciousness as a result of a cerebral tumor or hemorrhage is taken for granted, often without asking why it occurred in a particular case. It has been suggested in these cases, and we include meningiomas, that a direct or indirect disturbance of function of the diencephalon may be a determining factor (1). These effects are often present as a progressive deteriorating condition. However, the same clinical experience, had led to the differentiation of certain characteristics of the state of unconsciousness in relation to the site and nature of the causative lesion (1).

We cannot find in the literature an account of any proved mechanism explaining the rapid deterioration where hemorrhage and thrombosis is not a complication. Case 3 could fall within the category of upper brain-stem compression syndrome as described by Howell (10), except for the lack of extensor rigidity and a noisy respiration. It is possible that in cases 2 and 4 a direct compression at the "anterior critical point" of Jefferson were present. An "indirect" compression of this structure did occur in Case 1.

Jefferson (11, 12) says that in compression of the "anterior critical point" there is progression from drowsiness to coma either quickly or slowly with very little to show in the way of intracerebral hematoma, but frank infarction resulting from spasm of the vessels of the anterior perforated substance and accompanying edema. We assume that this edema, when progressive, will lead to the upper brain-stem compression syndrome of Howell, but this would need to develop quickly to account for rapid deterioration and loss of consciousness. Jefferson has stated that for the mechanism he describes, the brain-stem, the posterior hypothalamus, the basal ganglia and the cortex do not necessarily need to be damaged. His observation is based on clinical and pathological material. He has suggested too that, more frequently, stupor is caused very rapidly by massive lesions occuring anywhere in the cranial cavity, a common cause being pressure at

the tentorial level, his "posterior critical point" which corresponds to a critical level of the brain-stem reticular activating system.

It is not possible to arouse a patient by stimulating sensory tracts when he does not have a functional reticular formation (6), as in state of coma. There is an inclination to accept integration of different regions essential to consciousness such as the brain-stem, hypothalamus, region of the third ventricle, cerebral hemisphere. The evidence provided in man and animals of the fundamental mechanism of the reticular activating system in consciousness leads us to assume that the area of Jefferson's "anterior critical point" is closely related to that system. Experimental and more anatomical-clinical data are needed before this hypothesis can be accepted as fact.

Summary

Four cases of intracranial meningioma are reported which had normal skull x-rays and swiftly progressive loss of consciousness, suggesting rapid growing gliomas and various vascular lesions. The possible mechanism of unconsciousness in these cases is briefly discussed.

Acknowledgment

The authors wish to thank Dr. S. R. Snodgrass for the use of Case 3.

REFERENCES

1. Brain, Russell: The Physiological Basis of consciousness. *Brain* 81: 426–455, (September) 1958.
2. Cushing, Harvey: *Intracranial Tumours.* Notes upon a series of two thousand verified cases with surgical-mortality percentages pertaining thereto. Springfield and Baltimore. Charles C Thomas Publisher, 1932.
3. Cushing, H., and L. Eisenhardt: *Meningiomas.* Their classification, regional behaviour, life history and surgical end results. Springfield and Baltimore, Charles C. Thomas, Publisher, 1938.
4. Dechaume, J., P. Wertheimer, et P. Milleret: Reflexions sur une statistique de 114 meningiomes intracraniens. *Revue Neurologique* 81: 321–331 (Mai), 1949.
5. Fincher, E. F.: *Experiences with meningiomas.* The Surgical Clinics of North America, Toronto Number, pp. 1037–1049 (August), 1954.
6. French, L. J.: Anatomico physiologic Orientation to the Brain Stem. *Clinical Neurosurgery* (Proc. of the Congress of Neurological Surgeons) 6: 119–130, 1959.
7. Grant, F. C.: *Intracranial meningiomas.* Surgical results. Surg., Gynec., and Obst. 85: 419–431, (October) 1947.

8. Hoessly, G. F., and H. Olivecrona: Report on 280 cases of verified parasagittal meningiomas. *Journal of Neurosurgery* 12: 614–626, (November) 1955.

9. Horrax, G., and R. E. Strain: Subjective and Objective criteria in the diagnosis of meningiomas of the brain, with remarks to mortality and useful survival. *Annals of Surgery* 135: 892–898, (June) 1952.

10. Howell, D. A.: Upper brain-stem compression and foraminal impaction with intracranial space-occupying lesions and brain swelling. *Brain* 82: 525–550, (September) 1959.

11. Jasper *et al.*: *Reticular Formation of the Brain.* Boston, Toronto, Little Brown and Company, Publishers 1958, pp. 729–738.

12. Jefferson, Geoffrey.: The nature of concussion. *British Medical Journal* 1: 1–5, (January) 1944.

13. Moore, M. T.: The fate of clinically unrecognized intracranial meningiomas. *Neurology* 4: 837–856, (November) 1954.

14. Petit-Dutaillis, D., and S. Daum: *Les meningiomes de la fosse posterieure. Revue Neurologique* 81: 557–572, (Juliet) 1949.

ENZYMES OF THE KIDNEY OF THE NORMAL AND HYPERTENSIVE RAT*

MOHAMMED MATIUR RAHMAN, ARTHUR GROLLMAN, and LESTER PACKER

It is now generally accepted that the kidney is concerned in the pathogenesis of hypertension (9). The present study was undertaken, therefore, to compare the enzymatic functions of the normal and hypertensive kidney in order to determine if hypertension is associated with any changes in the enzymatic activity of the kidney. Although one cannot at present define the renal dysfunction in hypertension in terms of any single enzymatic process, the data obtained contribute to our understanding of renal enzymology which has been the subject of many recent studies on human as well as animal tissues. The present study was designed to investigate certain enzyme systems concerned in carbohydrate and phosphate metabolism, and energy production linked to cellular respiration, in order to compare the metabolic pattern of the kidney in the normal and hypertensive animal. A few determinations have been included on heart and liver since available data suggest that these also manifest differences in normal and hypertensive animals.

Materials and Methods

Male adult rats of the Long-Evans strain, reared in the laboratory, were used. Hypertension was induced by the "choline-deficiency" procedure of Grollman and White (10) which involves no operative procedure and results in renal changes comparable to those observed in essential hypertension of the human. The blood-pressures of the hypertensive animals, as determined by the method of Williams, Harrison and Grollman (27), ranged from 160 to 200 mms. Hg., as compared to 112 ± 14 mms. for the control normal rats. In all cases, the hypertension was of, at least, 3 months duration.

The animals were killed by dislocation of the cervical spinal cord and the tissues removed and placed in 0.25 M sucrose precooled to 3 to 5° C. Since the kidney is a heterogeneous organ, studies were made on its three major con-

* Supported by a Grant from the Public Health Service, National Institutes of Health (H-4124-C2). From the Departments of Experimental Medicine and Microbiology, The University of Texas Southwestern Medical School, Dallas 35, Texas. Received for Publication April 20, 1961.

stituents—the outer cortex, inner cortex and medulla—which were separated by dissection of the chilled tissue, as well as on the whole organ. Homogenates were prepared in a Potter-Elvehjem homogenizer with 10 volumes of 0.25 M sucrose. The crude homogenates were freed of gross particles by centrifuging in a refrigerated Sorvall Model SS-1 centrifuge, which was set at 3000 × g for a total of 30 seconds. A portion of the supernate was used immediately for manometric studies; part of the remainder was stored at — 15° C for subsequent determination of phosphatases. The remaining homogenate was centrifuged at 10,000 × g for 10 minutes and at 105,000 × g for one hour and the supernatant solution was used for the spectrophotometric determination of the pyridine nucleotide-linked enzymes. Kidney mitochondria and submicroscopic particles were separated, as described by Schneider (23), except that the precipitate obtained after centrifugation at 10,000 × g, suspended in ice-cold 0.25 M sucrose solution, was used to separate the mitochondrial particles. The mitochondria were washed twice with this solution.

Assay procedures. Reaction systems used for assay are listed in the tables. The manometric determinations were made in a Warburg respirometer at 37° C and the rates of oxygen utilization calculated from the time-uptake curves determined over a period of 1 hour. Polarographic studies of oxygen consumption were made as described by Packer (20). Spectrophotometric measurements of the pyridine nucleotide-linked enzymes were made on a Beckman DU spectrophotometer by the procedure of Kornberg and Horecker (16). The turnover numbers and molar concentrations of the mitochondrial respiratory chain were determined from difference spectra obtained with a Bausch and Lomb Spectronic 505 recording spectrophotometer. Fluorometric measurements were carried out in a Brice-Phoenix universal light-scattering photometer.

Malate and isocitrate oxidation rates were determined by the the methods of Ochoa (18, 19) at 25–26° C. Lactic dehydrogenase was assayed by the procedure of Kornberg (15).

Alkaline phosphatase was determined at pH 8.8 in a system containing β-glycerophosphate (33 mM), Tris (6.7 mM) and $MgCl_2$ (7 mM); acid phosphatase, at pH 5.0, by the method of Appelmans *et al.* (1), after incubation as suggested by Strauss (24); glucose-6-phosphatase, at pH 6.5, by the method of de Duve *et al.* (5); ATP-ase, at pH 7.4, by the method of Potter (21). Inorganic phosphate was determined by the method of Fiske and Subbarow (7); protein by the method of Kingsley (14), after solubilizing the tissue in 1 per cent deoxycholate. Bovine serum albumin (fraction V) was used as a standard.

The turnover numbers and concentrations of the respiratory components in mitochondria were determined, as described by Chance and Baltscheffsky (3), from the difference of the oxidized and the reduced spectra and the oxygen utilization.

The data were analyzed statistically as described by Fisher (6). Standard deviation of the means (S.D.) are expressed as $\pm \sqrt{\frac{\Sigma d^2}{n-1}}$. The differences of the means are reported only whenever statistically significant (P value less than 0.05).

Experimental Results

Citrate oxidation. The rate of oxidation of citrate by homogenates of whole kidney, outer and inner cortical areas of the kidney and of the heart are summarized in Table I. It may be noted that oxygen utilization is greatest in the inner cortex reflecting the presence of proximal and distal tubular segments in this area. The medulla of the kidney, on the other hand, (data for which are not included in the table) consumed citrate only slowly (61.9 ± 9 μl. of O_2 per hour per mg. protein), and the rate was increased only slightly by supplementation with acetate (2 mM) and pyruvate (2 mM). In this respect the medulla resembles the heart. As noted in Table 1, there is a reduction in

TABLE 1

Rates of oxidation of citrate in kidney and heart of 3 normal rats as compared to 8 hypertensive animals.

The reaction system (3 ml.) at pH 7.4, contained KH_2PO_4 (1 mM), $MgCl_2$ (5 mM), ATP (1 mM), citrate (13.3 mM), Tris (7 mM) and sucrose (200 mM). Results are expressed as μl of O_2 consumed per hour per mg. protein.

	Whole kidney				
	Without substrate	With substrate	Outer cortex	Inner cortex	Heart*
Normal	34.2±4.5	225.6±9.0	133.8±7.5	168.6±9.0	66.0±6.0
Hypertensive	30.9±6.0	203.7±6.0	114.6±9.6	136.2±10.2	57.0±7.8
Difference (per cent)		9.7	14.4	19.2	
P value		<0.05	<0.01	<0.01	

* Supplemented with acetate (2 mM) and pyruvate (2 mM).

citrate oxidative capacity in the kidney of the hypertensive as compared to the normal in tissue homogenates supplemented by substrate, as suggested by Potter (21).

It should be noted in Table 1 that there is a discrepancy between the rate of oxygen utilization by whole kidney and its constituent parts. This is not a result of loss of activity induced by separation of these parts, for similar results were obtained when the homogenates were prepared from the separate portions of the whole kidney and then recombined. The cause of this phenomenon is unknown and may have a bearing on the differences in the values obtained on the normal and hypertensive tissues.

Pyridine nucleotide-linked enzymes. Since the enzymes involved in the oxidation of GP* and PG are present in the soluble portion of the supernatant fluid of tissue homogenates (8), this system was utilized for study of these pyridine nucleotide-linked enzymes.

The data of Table 2 do not reflect the true GP-dehydrogenase content as the PG-dehydrogenase present in the tissue also oxidizes PG produced by oxidation of GP. However, when PG is used as substrate, the observed activity reflects the PG-dehydrogenase content (4).

As shown in Table 3, there is a reduction in the malic dehydrogenase but an increase in the isocitric dehydrogenase of the renal cortex in the hypertensive as compared to the normal kidney. There is also a statistically significant increase in the isocitric dehydrogenase of the heart and lactic dehydrogenase activity of the outer cortex. As shown in Table 2, there are no differences between the GP and PG activities of the liver and heart of normal as compared to hypertensive animals. The kidney, on the other hand, manifests: (1) a higher GP activity of the outer cortical tissue in the hypertensive as compared to the normal; (2) a lower GP activity of the inner cortical tissue in the hypertensive as compared to the normal; (3) no statistically significant differences in PG activity in any portions of the kidney of the normal as compared to the hypertensive animal; (4) a markedly reduced PG and GP activity in the medullary as compared to the cortical tissues; and (5) a greater ratio of the rate of oxidation of GP to PG for the various portions of the kidney as compared to the heart and liver.

Phosphatases. The alkaline, acid and GP phosphatases and ATPase contents of homogenates of the inner and outer renal cortex are given in Table 4. The results confirm the well-established high alkaline phosphatase content of the kidney (26) and show a slight reduction in this activity in the hypertensive.

* The following abbreviations are used throughout this paper:
ATP, Adenosine triphosphate
DPN, Diphosphopyridine nucleotide
DPNH, Reduced diphosphopyridine nucleotide
EDTA, Ethylenediaminetetracetic acid
GP, Glucose-6-phosphate
PG, 6-Phosphogluconate
Pi, Inorganic phosphate
TPN, Triphosphopyridine nucleotide
Tris, Tris (hydroxymethyl) aminomethane

TABLE 2

Rates of reduction of triphosphopyridine nucleotide by glucose-6-phosphate and 6-phosphogluconate by supernatants of tissues from 11 normal and 6 hypertensive rats.

Reaction system (3.0 ml.) contained $MgCl_2$ (13 mM), TPN (10^{-4} M), glycylglycine (6 mM) and substrate (1.3 mM). Results are expressed in terms of change in optical density at 340 mμ per minute, per mg. protein.

	Outer cortex			Inner cortex			Medulla			Heart			Liver		
Substrate	GP	PG	Ratio	GP	PG	Ratio	GP	PG	Ratio	GP	PG	Ratio	GP	PG	Ratio
Normal	137.0	99.5	1.4	170.6	80.2	2.1	65.4	18.4	3.6	34.9	29.5	1.2	109.3	92.4	1.2
S.D.	±4.5	±6.0	±0.1	±9.0	±7.5	±0.1	±10.5	±4.5	±0.2	±3.0	±3.0	±0.2	±6.0	±4.5	±0.1
Hypertensive	150.0	102.8	1.5	135.0	75.7	1.78	63.4	17.8	3.6	34.5	29.1	1.2	107.2	93.1	1.2
S.D.	±4.5	±3.0	±0.02	±7.5	±6.0	±0.1	±6.0	±3.0	±0.3	±4.5	±3.0	±0.1	±4.5	±3.0	±0.1
Difference (per cent)	+9.5		+5.8	20.9		+16.4									
P value	<0.01		<0.05	<0.01		<0.01									

TABLE 3

The activity of pyridine nucleotide-linked enzymes in normal and hypertensive rats.

Malic and isocitric oxidation rates are expressed as differences in optical density at 340 mμ, per minute, per mg. protein; lactic dehydrogenase activity is expressed as μmole DPNH oxidized per 100 mg. protein. The averages ± S.D. of data from 5 animals are included with differences expressed in per cent and P values where significant.

	Malic enzyme			Isocitric enzyme			Lactic dehydrogenase		
	Normal	Hypertensive	Difference	Normal	Hypertensive	Difference	Normal	Hypertensive	Difference
Outer cortex	7.7	5.9	—23.3	68.4	85.2	+24.3	124.8	134.1	+7.4
	±0.6	±0.9	P<0.01	±7.5	±6.6	P<0.05	±6.6	±6.0	P<0.05
Inner cortex	7.4	5.8	—21.5	69.9	84.9	+21.3	136.8	144.3	
	±0.7	±0.6	P<0.01	±6.0	±6.3	P<0.05	±6.3	±6.9	
Medulla	2.2	2.3		33.9	36.6		54.9	53.7	
	±0.3	±0.3		±4.5	±3.6		±4.8	±3.0	
Heart	5.2	5.2		172.2	207.3	+20.3	470	476	
	±0.3	±0.3		±9.6	±11.1	P<0.01	±27	±15	
Liver	12.2	12.6		347.7	351.6		671	662	
	±0.9	±1.1		±16.5	±18.0		±38	±32	

TABLE 4

Phosphatase in homogenates of the kidney from normal and hypertensive rats.

Results are averages of determinations on 5 rats expressed as μg of inorganic phosphate liberated from substrate per hour.

	Alkaline phosphatase		Acid phosphatase		Glucose-6-phosphatase		ATPase	
	outer cortex	inner cortex	outer cortex	inner cortex	outer cortex	inner cortex	outer cortex	inner cortex
Normal	318	320	164	168	218	233	183	180
S.D.	±12	±16	±12	±11	±12	±13	±7	±12
Hypertensive	298	292	165	165	215	247	185	190
S.D.	±8	±12	±10	±9	±11	±11	±10	±14
Difference (per cent)	—6.5	—8.7				+6		
P value	<0.05	<0.05				<0.05		

On the other hand, the glucose-6-phosphatase activity of the inner cortex was slightly higher in the hypertensive as compared to the normal. The inorganic phosphate in the original homogenates was essentially the same (5.1 ± 0.3 μg) per mg. protein in the normal as compared to the hypertensive animals (5.2 ± 0.5).

Respiration and phosphorylation of mitochondria. The integrity of the respiratory chain and phosphorylating systems in hypertension is reflected in the data of Tables 5–7. The Q_{O_2}

TABLE 5

Average turnover numbers and relative concentrations of the respiratory components in the mitochondria of the kidneys from two normal and hypertensive rats.

Reaction system contained succinate (10 mM) as substrate, sucrose (50 mM), $MgCl_2$ (5 mM), KCl (40 mM) and phosphate (as K salt, 20 mM) at pH 7.5.

Component	Turnover number		Concentration		Relative concentration	
	Normal	Hypertensive	Normal μmoles per mg. protein $\times 10^{-4}$	Hypertensive	Normal	Hypertensive
Cytochrome a	9.05	7.64	3.1	3.3	1.0	1.0
Cytochrome b	21.65	20.76	1.3	1.2	0.4	0.4
Cytochrome c	5.33	5.01	5.3	5.0	1.7	1.5
Flavoprotein	7.21	5.30	4.1	5.2	1.3	1.6
Cytochrome a_3	8.85	8.00	3.2	3.2	1.0	1.0
Pyridine nucleotide	2.75	3.63	10.2	7.1	3.5	2.2

TABLE 6

Oxygen utilization by mitochondria of normal and hypertensive kidneys.

Reaction system (3 ml.) at pH 7.4, contained phosphate (K-salt, 20 mM), $MgCl_2$ (5 mM) and sucrose (100 mM) with α-ketoglutarate (10 mM) as substrate. Results are averages on 4 animals and are expressed as μl of O_2 per hour, per mg. protein. The β-hydroxybutyrate was supplemented with ATP (2 mM) and DPN (2 mM); the lactate was supplemented with DPN (2 mM).

Substrate	Choline	β-hydroxybutyrate	Lactate
Normal	67.5±7.8	92.7±7.5	88.5±7.8
Hypertensive	69.3±10.2	96.3±6.3	96.9±5.1

TABLE 7

Oxidative phosphorylation and ATPase activity in mitochondria from kidney of normal and hypertensive rats.

Reaction system (3.0 ml.) at pH 7.4, contained α-ketoglutarate (10 mM), malonate (10 mM), phosphate (K-salt, 20 mM), $MgCl_2$ (5 mM), ADP (1 mM), sucrose (100 mM) and EDTA (0.5 mM), hexokinase (320 Km Michaelis units) and glucose (150 μmoles). Average results on 3 rats are expressed as μ Atoms of oxygen and μmoles of Pi per hour per mg. protein per ml. ATPase activity was determined in the reaction system described by Potter (21).

	O_2 utilization	Pi-consumption	ATPase activity
Normal	11.3±0.1	35.6±3.0	11.9±0.2
Hypertensive	12.1±1.8	37.5±5.4	11.7±0.3

values with succinate were determined polarographically as described by Packer (20). These values were 0.70 ± 0.05 and 0.69 ± 0.02 μM per second per mg. of mitochondrial protein, respectively, for the normal and hypertensive kidneys. Both showed respiratory control ratios of 2 with 200 μM ADP. As noted from Table 5 the mitochondria from normal and hypertensive kidneys show the same patterns of turnover numbers and relative concentrations of the components of the respiratory chain, and there is no evidence of any functional deficiency in the respiratory chain. Similar results for the concentration of the cytochromes were obtained using dithionite as reducing agent instead of reducing these pigments enzymatically. Our results are in contrast to the conclusion of Raska (22) that the cyto-

chrome oxidase and cytochrome c content of the kidney of dogs rendered hypertensive by application of a Goldblatt clamp is reduced.

A comparison of the data of Table 5 with the results of Chance and his co-workers (3) indicates that the relative concentration of cytochrome $c + c_1$ in kidney mitochondria is near that from liver or heart mitochondria. On the other hand, the relative concentration of flavoprotein and pyridine nucleotide in kidney mitochondria is similar to that in heart, but less than in mitochondria of the liver.

To induce oxidation of glutamate, kidney mitochondria required the presence of DPN. The oxidation of β-hydroxybutyrate and lactate was stimulated, 20 and 35-fold, respectively, by such supplementation and the substrates were therefore examined in the presence of added cofactor (Table 6). The oxidation of these substrates as well as of choline by mitochondria was not altered in the hypertensive as compared to the normal kidney.

The phosphorylating capacity of mitochondria, as shown in Table 7, is also not altered in the hypertensive animal. The values for inorganic phosphate consumed, the ratio of phosphate to oxygen consumption and the ATPase activity were the same in normal and hypertensive animals.

Discussion

Several investigators have concluded that hypertension is associated with such specific alterations in enzyme function as a reduction in the oxidative metabolism of the kidney (2, 17, 22). The reduction of glucose-6-phosphate dehydrogenase content of the macula densa cells of the tubules, as observed histochemically, is particularly striking (12). The present study also reveals certain enzymatic patterns in the kidney. The internal cortical area, which contains the active proximal and distal portions of the tubules, manifests greatest activity as regards citrate and glucose-6-phosphate oxidation; the outer cortex oxidizes phosphogluconate at the most rapid rate. The metabolism of pyridine nucleotides, as reflected in studies of malic, isocitric and lactic dehydrogenases, is less active than that observed in heart and liver. On the other hand, the participation of flavoprotein and pyridine nucleotides in the respiratory chain of kidney mito-

chondria resembles that of heart mitochondria and is of a lesser intensity than in the liver.

The data presented demonstrate the marked differences in enzymatic activity manifested by the different areas of the kidney and the necessity for compartmental analysis. This is not unexpected in view of the demonstrated differences in function of the component parts of the kidney. In the normal kidney, enzymatic activity is appreciably less in the medulla as compared to the cortex in the case of all the enzyme systems examined. The citrate and lactic dehydrogenase and the TPN-linked GP dehydrogenase enzymes are approximately 20 per cent greater in the inner as compared to the outer zone of the cortex. On the other hand, the TPN-linked PG dehydrogenase is less in the inner than in the outer cortex. Other enzymic moities such as DPN-linked malic dehydrogenase and the four phosphorylitic enzymes are present in equal amounts in both portions of the cortex.

It is significant that the pattern of enzyme distribution in the hypertensive kidney is essentially the same as in the normal. However, certain differences are observed between the hypertensive and normal kidneys, *viz.*: (1) a decreased rate of oxidation of citrate and an increased rate of isocitrate oxidation in both areas of the cortex in the hypertensive as compared to the normal; (2) a lesser increase in lactic dehydrogenase activity in the hypertensive; (3) an increase in the TPN-linked GP activity of the outer zone with a decreased activity in the inner zone; and (4) the same rate of PG activity in all portions of the hypertensive kidney as compared to the normal. These differences in TPN-linked enzymes suggest changes in the pathways of glucose utilization.

Of the four phosphatases examined only the alkaline phosphatase was significantly lower (in both the inner and outer cortex) and the glucose-6-phosphatase was higher (in the inner zone only) in the kidney of the hypertensive as compared to the normal animal.

The respiratory enzymes of kidney mitochondria from normal and hypertensive animals show a similar pattern of activity. Such differences as were noted cannot be regarded as significant. Likewise, the oxidation of substrate (Table 6) and oxidative

phosphorylation of α-ketoglutarate are similar in the kidneys from both groups of animals.

Although suggestive, the changes in enzymatic function observed in hypertension do not permit one to conclude that these necessarily reflect a molecular basis for this disorder. It is significant that such major biochemical processes at the cellular level as are concerned in energy synthesis and mitochondrial function are essentially the same in the hypertensive and normal animal. The presence of such differences as were observed does not imply necessarily that these are the specific causes of hypertensive disease since the procedures used for inducing hypertension might by their incidental morphologic disturbances be responsible for the observed changes in enzymatic function. This is particularly true of previously reported studies which were carried out on animals in which hypertension was induced by operative manipulation of the kidney with gross morphologic disturbance of the renal parenchyma.

Summary

Enzyme systems concerned in the energy synthesis of mitochondria, *viz.*, the respiratory chain and oxidative phosphorylation, and the activities of the pyridine nucleotide dehydrogenases were compared in the kidneys, heart and liver of normal and hypertensive rats. Certain statistically significant differences in activity were found including a reduction in rate of citrate oxidation, alkaline phosphatase, and malic dehydrogenase activities and an increase in isocitric and lactate oxidation and GP-ase activities in the tissues of hypertensive as compared to normal animals. However, the general pattern of the major biochemical processes at the cellular level, such as phosphate esterification by respiratory chain phosphorylation, phosphate-ester breakdown by phosphatases, the components of the mitochondrial and respiratory chain, and the activities of pyridine nucleotide-linked enzymes, are quantitatively similar in normal and hypertensive animals.

REFERENCES

1. Appelmans, F., R. Wattiaux, and C. de Duve: Tissue fractionation studies. *Biochem. J.*, 59: 438, 1955.

2. Bierwagen, M. E., and D. L. Smith: Dehydrogenase enzyme studies in experimental renal hypertension. *Proc. Soc. Exp. Biol. Med.*, 95: 581, 1957.
3. Chance, B., and M. Baltscheffsky: Effects of ADP in heart muscle sarcosome. *Biochem. J.*, 68: 283, 1958.
4. Dickens, F.: The significance of direct pathway for glucose oxidations. Symposia of Brookhaven National Laboratory, No. 5. U. S. Government Printing Office, Washington, 1952, p. 134.
5. de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans: Tissue fractionation studies. *Biochem. J.*, 60: 604, 1955.
6. Fisher, R. A.: Statistical Methods for Research Workers, 12 ed., Hafner Publishing Co., Inc., New York, 1954, p. 114.
7. Fiske, C. H., and Y. Subbarow: The colorimetric determination of phosphorus. *J. Biol. Chem.*, 66: 375, 1925.
8. Glock, G. E. and P. McLean, Glucose-6-phosphate dehydrogenase activity in rat liver. *Biochem. J.*, 55: 400, 1953.
9. Grollman, A.: A unitary concept of experimental and clinical hypertensive cardiovascular disease. *Perspectives in Biol. & Med.*, 2: 208, 1959.
10. Grollman, A., and F. N. White: Induction of hypertension in rats and dogs by potassium or choline deficiency. *Amer. J. Physiol.*, 193: 144, 1958.
11. Hess, R.: The histochemical analysis of enzymatic processes in the renal tubule. *in* Diuretics, E. Buchborn and K. D. Bock, Eds., Springer-Verlag, Berlin, p. 121, 1959,
12. Hess, R. and F. Gross: Glucose-6-phosphate dehydrogenase and renin in kidneys of hypertensive and adernalectomized rats. *Am. J. Physiol.*, 197: 869, 1960.
13. Huckabee, W. E.: Inadequacy of renal oxygen in essential hypertension. *J. Clin. Invest.*, 37: 903, 1958.
14. Kingsley, G. R.: Determination of serum total protein, albumin and globulin by biuret reaction. *J. Biol. Chem.*, 131: 197, 1939.
15. Kornberg, A.: Lactic dehydrogenase of muscle. Ref. 16, p. 441.
16. Kornberg, A., and B. L. Horecker: Glucose-6-phosphate dehydrogenase, *in Methods* in Enzymology, S. P. Colowick and N. O. Kaplan, Eds. Academic Press, New York, 1: 323, 1955.
17. Lamperi, S., and G. Cambriaggi: Enzyme activity of kidney tissue in experimental hypertension. *Arch. E. Maragliano patol. e chimie*, 10: 1, 1955.
18. Ochoa, S.: Isocitric dehydrogenase system from pig heart and malic enzyme. Ref. 16, p. 699.
19. Ochoa, S.: Malic enzyme. Ref. 16, p. 739.
20. Packer, L.: Regulation of respiration in heart muscle sarcosomes. *Exptl. Cell. Res.*, 15: 551, 1958.
21. Potter, V. R.: The homogenate technique, *in* Manometric Techniques, W. W. Umbreit, R. H. Burris and J. F. Stauffer, Eds. Burgess Publishing Co., Minneapolis, 1959, p. 170.
22. Raska, S. B.: Disturbed metabolism in hypertensive kidney. *J. Exptl. Med.*, 82: 227, 1945.
23. Schneider, W. C.: Methods for the Isolation of Particulate Components of the Cell, in *Manometric Techniques*. W. W. Umbreit, R. H. Burris, and J. F. Stauffer, Eds. Burgess Publishing Co., Mineapolis, 1959, p. 188.

24. Strauss, W.: Localization of acid phosphatase in the kidney. *J. Biochem. Biophys. Cytology*, 2: 513, 1956.

25. Taggart, J. V.: Some biochemical features of tubular transport mechanism in the kidney. Ciba Foundation Symposium on the Kidney, A. A. G. Lewis and G. E. W. Wolstenholme, Eds., Little, Brown & Co., Boston, 1954, p. 65.

26. Utter, M. F.: The role of carbon dioxide fixation in carbohydrate utilization and synthesis. *Ann. N. Y. Acad. Sci.*, 72: 451, 1958.

27. Williams, J. R., Jr., T. R. Harrison, and A. Grollman: A simple method for determining the systolic blood pressure of the unanesthetized rat. *J. Clin. Invest.*, 18: 373, 1939.

EFFECT OF MORPHINE ON PITUITARY CYTOLOGY AND GONADOTROPHIC LEVELS IN THE RAT[a]

EDWARD G. RENNELS[b]

Introduction

In a preliminary report it was pointed out that the daily injection of morphine sulfate into young female rats for a two week period results in a threefold increase in the pituitary content of FSH (follicle stimulating hormone) (Rennels, 1960). Since this treatment did not alter the pituitary level of LH (luteinizing hormone) it was of interest to study the effect of morphine treatment on the gonadotrophic cells of the pituitary gland. We have shown earlier that two tinctorial types of gonadotrophs can be differentiated in the rat by the use of the PAS-methyl blue stain (Rennels, 1957) and evidence that these two cell types are responsible for the elaboration of FSH and LH has been presented (Hildebrand, *et al.* 1957; Hellbaum, *et al.* 1961). In the present paper a detailed account of the hormonal assays and the cytological observations of the pituitary will be given.

Materials and Methods

The rats used in this study were all obtained from the Holtzman Rat Company. Those which were treated with morphine sulfate were given daily subcutaneous injections (60 mg./kg.) for a two week period beginning at an age of 47 days. Although the initial experiments involved only female rats the study was later extended to include males of the same age.

Vaginal smears were made on all of the females beginning at an age of 45 days. At this age there has been at least one spontaneous ovulation and the vaginal membrane has already ruptured. All morphine injections were given between noon and 2 p.m. in order that a blockade of ovulation might be achieved (Barraclough and Sawyer, 1955). The results reported in this paper are based on pituitary material taken from 5 series of female rats and two series of males. Pituitary glands from most of the animals studied were used in the bio-assays for LH and FSH. At autopsy the pituitary glands were quickly removed and either prepared for bio-assay or fixed for cytological study.

[a] This study was supported by research Grant RG 4723 from USPHS. Submitted for publication May 2, 1961.

[b] From the Department of Anatomy, The University of Texas Medical Branch, Galveston, Texas.

In the preparation of glands for bio-assay the posterior lobe was separated and discarded. The anterior lobe was then weighed on a torsion balance and placed between two clean microscopic slides. Pressure was applied to the slides causing the pituitary tissue to spread into a thin film. The two slides were separated and placed in desiccator jars for drying. A few days later the dried material was scraped from the slides, weighed and placed in ampules for later administration to assay animals.

Pituitaries fixed for cytological study were placed in either Dawson's fluid (saturated mercuric chloride in saline, 9 parts; formaldehyde, 1 part) or 10% formol-saline. After processing and sectioning, representative sections from control and experimental glands were mounted together on slides and stained with PAS-methyl blue or aldehyde-fuchsin. Details of the mounting procedure and modifications of the staining methods used have been published previously (Rennels, 1957).

Ovaries from control and morphine-treated animals of one series were taken at autopsy, weighed and fixed in Dawson's fluid. Paraffin sections were prepared and stained with hematoxylin and eosin. Routine H and E sections of testes and ventral prostate were prepared in the case of the male series.

Assays for FSH and LH were performed on the air-dried pituitary material. For the assay of LH the ventral prostate method (Greep, *et al.* 1941) utilizing immature hypophysectomized male rats was employed. Holtzman male rats were hypophysectomized at 22 days of age. Beginning on the second day after hypophysectomy 4 daily subcutaneous injections of pituitary material were given and the animals were autopsied on the day following the last injection. The dried pituitary material was suspended in distilled water and kept under refrigeration between injections. At autopsy the testes, ventral prostate, and adrenal glands were weighed on a torsion balance. In addition, a careful check of the sellar region was made for pituitary fragments. The assay animals were not included if pituitary fragments were found or if the adrenal weight indicated the presence of a functional remnant of anterior lobe tissue.

The FSH content of the dried pituitary material was determined by the use of the assay described by Steelman and Pohley (1953). In this assay 21-day-old intact female rats are given daily intraperitoneal injections of 10 IU human chorionic gonadotrophin (HCG) for 3 days. This treatment produces an ovarian stimulation which can be augmented by the additional injection of FSH or pituitary material containing FSH. Preliminary assays indicated that the pituitary material from the morphine-treated animals was far more potent than that from the control animals and in later assays the dose was adjusted so as to obtain essentially a matched response with these two preparations. All injections of pituitary material were given by the subcutaneous route and in all assays uninjected control groups as well as HCG control groups were included. At autopsy on the day following the last injection the ovaries were dissected and weighed.

All assays reported were performed on at least two different series of assay animals. Due to the greater gonadotrophic potency of the male pituitary it was necessary to reduce the dosage given the assay animals, especially in the

assay of FSH. In analyzing the data the "t" test of significance was used in the comparisons of certain mean values.

Results and Discussion

The effects of morphine treatment on the body weight and weight of the anterior pituitary gland are shown in Table 1. Both

TABLE 1

Effects of morphine treatment on young adult female rats

Treatment	No. persistent diestrus	Body weight gm.	Anterior pituitary mg.
None-controls	0/45	178±1.6*	7.01±0.14
Morphine sulfate†	27/62	159±1.4	5.35±0.10

* Standard error.
† Subcutaneous injections were given daily for 14 days commencing on the 47th day of age. The injections were given between noon and 2:00 P.M. at a dosage of 60 mg./kg.

weights are significantly lower in the morphine-treated group ($P = < .001$) and the suppression in pituitary weight is more than a reflection of reduced body weight. The lower body weight can be explained in part, at least, on the basis of a reduced intake of food and water during the treatment period. However, it has been shown in experiments involving pair-fed controls that the effects of morphine on the ovarian cycle are not a result of undernutrition (Barraclough and Sawyer, 1955). Behavioral signs (depression of reflexes, muscular incoordination and catatonia followed by hypertonicity and salivation) similar to those observed by Barraclough and Sawyer (1955) were seen in the animals following the daily injections. Table 1 also shows a summary of the effects observed on vaginal smears in two series of animals. All of the 45 normal females showed either normal cycles or no marked deviation from normal cycles while almost one-half of the morphine-treated animals exhibited diestrous smears for the 14 day treatment period. Most of the other treated animals showed abnormal cycles with a tendency toward a prolonged diestrous period. For assay purposes the pituitary material from the treated animals was divided into two pools; one contained the glands from the persistent diestrous animals while the other contained the glands from the animals which exhibited irregular cycles. The glands from the control animals were also grouped according to whether the animals showed estrous (and

proestrous) smears on the day of autopsy or diestrous smears. Tables 2 and 3 give the results of the hormonal assays of the

TABLE 2

Effect of morphine on pituitary content of LH in female rats

Pituitary donor group	No. assay animals†	Ventral prostate mg.
None-(uninjected controls)	7	10.9±1.0*
Normal females—estrus	8	31.5±2.2
Normal females—diestrus	10	36.1±2.0
Morphine treated females—persistent diestrus	10	36.3±1.3
Morphine treated females—irregular cycles	10	32.6±3.0

* Standard error.
† Assay animals were 22 day-old, hypophysectomized male rats. Each animal was given a total of 1 mg. of dried pituitary material.

TABLE 3

Effect of morphine on pituitary content of FSH in female rats

Pituitary donor group	No. assay animals†	Total dose mg.	Ovaries mg.
None-(uninjected controls)	13	. .	19.7±0.7*
None-(30 IU HCG only)	14	. .	39.7±2.3
Normal females—estrus	12	3.0	57.8±3.9
Normal females—diestrus	8	3.0	68.1±11
Morphine treated females—persistent diestrus	11	1.0	69.6±6.1
Morphine treated females—irregular cycles	11	1.0	48.0±2.9

* Standard error.
† Assay animals were 21 day-old intact, female rats. All groups given pituitary material received, in addition, 30 IU of HCG.

pituitary material obtained from the female rats. No difference in the pituitary content of LH was found on comparing the response of the ventral prostate to pituitary material from control and morphine-treated rats. Pituitary material from control animals killed in diestrus gave an almost identical response to that obtained with the pituitary material from morphine-treated rats killed in diestrus (36.1 compared to 36.3). In the case of pituitary material from both control and morphine-treated animals there is a suggestion that the glands may contain more

LH in the diestrous period than at other times, although the differences are not significant statistically. The testes were stimulated in all of the assay groups although these weights are probably not a good index of either LH or FSH content alone. It can be concluded from the LH assay that the morphine treatment did not affect the pituitary content of this hormone.

Results of the FSH assay are given in Table 3. It should be apparent that the pituitary material from the morphine-treated rats is approximately three times as potent as that from the control animals. In the case of the pituitary material from the diestrous donors one milligram of dry material from the morphine-treated rats gave an ovarian weight slightly above that obtained with three milligrams of material from control animals. A significant augmentation of the ovarian response produced by HCG alone was obtained in all assay groups which received, in addition, injections of pituitary material. Such an augmentation is considered to be a reflection of the FSH content of the injected pituitary material (Steelman and Pohley, 1953). Again, as in the case of the LH assay, it was noted that a greater response was obtained with the pituitaries from animals killed in diestrus; this was true for pituitary material from both the morphine-treated and control animals.

It was concluded from these assays that while the level of pituitary LH was not affected by the morphine treatment a marked increase in the pituitary content of FSH occurred. This information does not allow one to speculate on the effects which morphine may have on the *release* of either hormone but does offer an opportunity to relate the observed increase in pituitary content of FSH to any alterations which may occur in the gonadotrophic cells.

Students of rat pituitary cytophysiology now generally agree that by suitable staining methods a cytological differentiation can be made between those basophiles which manufacture gonadotrophic hormones (cells which are termed gonadotrophs or delta cells) and those which are concerned with the formation of thyrotrophic hormone (thyrotrophs or beta cells). Furthermore, several workers have presented evidence that two distinct categories of gonadotrophic cells exist in the rat, one of these being regarded as the FSH producer, the other as the LH producer. There is not yet agreement, however, as to the proper

identity of these two types of gonadotrophs in terms of their distribution pattern, staining characteristics, and fine structure. (See discussion by Hellbaum, *et al.* 1961). Using the PAS-methyl blue stain after formol-saline fixation, a color distinction can be made between the PAS-red gonadotrophs and PAS-purple gonadotrophs (Rennels, 1957). On the basis of changes in FSH and LH after castration the conclusion seemed inescapable that the PAS-red gonadotrophs were responsible for LH storage (and presumably production) and the PAS-purple type with FSH storage (Hildebrand, *et al.* 1957). Since this interpretation differs markedly from that arrived at by Purves and Griesbach (1954) in similar studies we have continued to seek additional evidence relative to the functional activities of these two tinctorial types of gonadotrophs. It was of particular interest, therefore, to analyze the pituitary glands of the morphine-treated animals for alterations in the PAS-reactive cells.

The gonadotrophs in the glands of the normal females were found to be essentially as previously described (Rennels, 1957). Both PAS-red and PAS-purple types were present although the total number of gonadotrophs present in the female gland at 60 days was quite small compared to the number seen in male glands. In the PAS-methyl blue preparations there was some difficulty in distinguishing the centrally placed gonadotrophs from the thyrotrophs which also react with the PAS reagent. This distinction could be made readily in sections stained with aldehyde fuchsin in which the thyrotrophs are selectively stained with the aldehyde fuchsin. A disadvantage of the latter method, however, is that both types of gonadotrophs are stained with the light green counterstain.

In the glands of females treated with morphine, no striking difference was found in the number, size, or staining qualities of the gonadotrophs. A slightly greater number of PAS-purple cells was observed in several glands but in others no clear-cut deviation from normal was seen. No attempt to do cell counts was made since it was felt that the lack of a random distribution together with the known overlap in the distribution patterns of the two types of gonadotrophs would make a meaningful analysis of such data almost impossible.

Since the pituitary of male rats is normally higher in FSH content and contains a much greater number of gonadotrophs than

the female it was considered advisable to study the glands of a group of male rats subjected to a similar period of morphine treatment. When compared to the glands of control animals it was found that the pituitaries of morphine-treated male rats showed a marked increase in number and size of certain of the gonadotrophic cells. The cells showing the most marked changes were those centrally placed, PAS-purple gonadotrophs which are considered on other evidence to be responsible for FSH elaboration (Figs. 1 and 2). These cells are distinguished from thyrotrophs (which are present in the same areas) not only by their staining affinities but also by shape, size, and positional relationship to vessels. A considerable variation in the number of thyrotrophs was observed among different individuals of both the normal and morphine-treated groups. In general, there seemed to be a somewhat greater number of thyrotrophs in the glands of the morphine-treated animals. This fact cannot have influenced our evaluation of the changes in gonadotrophs, however, since thyrotrophs stand out quite clearly in preparations stained with aldehyde fuchsin. In such preparations from morphine-treated animals the increased number of gonadotrophs in the central areas

EXPLANATION OF FIGURES

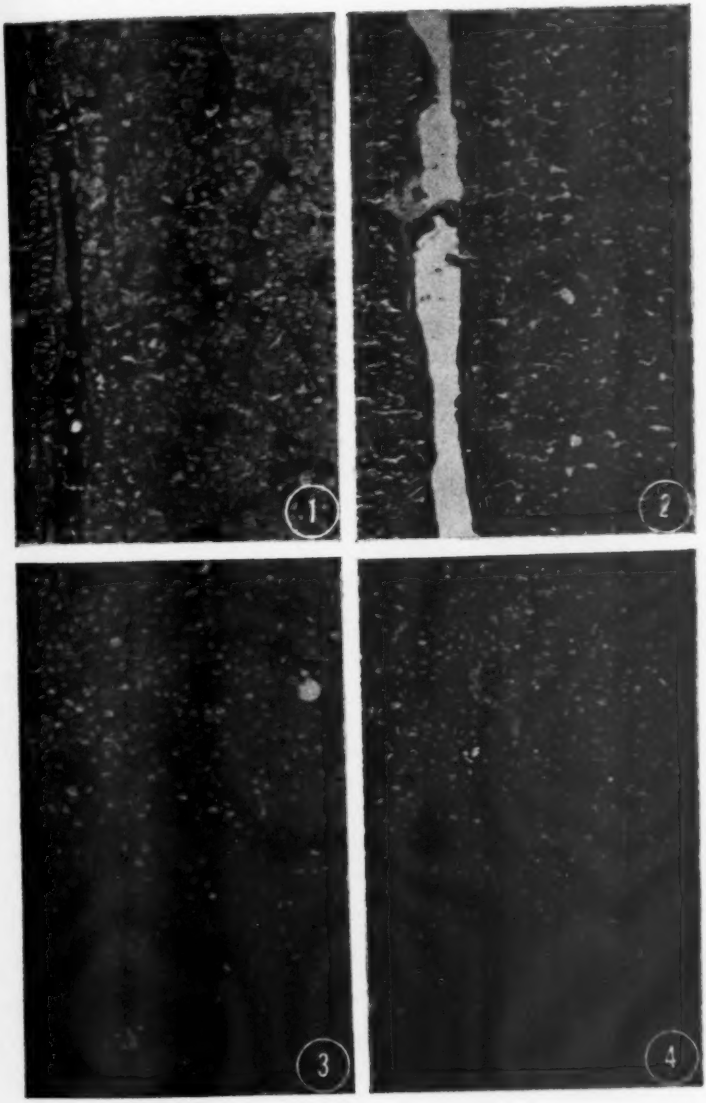
All figures are micrographs of 4 μ horizontal sections of male rat pituitary glands. The sections from which figures 1 and 2 were made were mounted together and stained with the PAS-methyl blue procedure, following fixation in 10% formol-saline. Sections from which figures 3 and 4 were prepared were mounted together and stained with the aldehyde fuchsin method after fixation in Dawson's fluid.

FIG. 1. Pituitary section of a normal male rat. A portion of the pars intermedia is shown on the left. This represents an area in which the PAS-purple gonadotrophs are normally located. Scattered cells of this type may be seen along the sinusoids. x230.

FIG. 2. Pituitary section from a morphine-treated male rat. The level and area shown are comparable to that shown in figure 1. Pars intermedia and residual lumen are seen on the left. Note the increased number and prominence of the PAS-purple gonadotrophs. Nearly all of the PAS-reactive cells in this field are PAS-purple gonadotrophs. x230.

FIG. 3. Pituitary section from a normal male rat. This field is of a central area which normally contains both thyrotrophs and gonadotrophs. Two thyrotrophs (dark purple) are seen in this field as well as scattered, round gonadotrophs which stained with the light green counterstain. Acidophiles are only faintly stained with orange G. x230.

FIG. 4. Pituitary section from a morphine-treated male rat. The level and area shown are comparable to that seen in figure 3. At the top and in the lower right corner are several thyrotrophs. Note the marked increase in number, size and staining intensity of the gonadotrophs, which are stained with light green. These centrally placed gonadotrophs are the PAS-purple type when stained with the PAS-methyl blue procedure. x230.


1
2
3
4

of the glands was apparent on inspection. At higher magnification it was seen that the centrally-placed gonadotrophs were not only more numerous but were also enlarged and more intensely stained (Figs. 3 and 4). Only very minor differences were found in the peripherally situated PAS-red gonadotrophs which are normally concentrated in the angle adjacent to the intermediate lobe and at the periphery of the gland.

It is known that the pituitary gland of the normal male rat contains a preponderance of FSH (Hellbaum and Greep, 1940), but it was necessary to determine if the effect of morphine on FSH storage was similar to that found in the female. For this purpose assays for LH and FSH were carried out on pituitary material from normal and morphine-treated males. The results of these assay are given in Tables 4 and 5. In regard to LH it was found that a somewhat greater amount of this hormone was present in male pituitaries since a total dose of 0.75 mg. of air-dried material from control males gave ventral prostate weights somewhat greater than was obtained with 1.0 mg. of pituitary material from con-

TABLE 4

Effect of morphine on pituitary content of LH in male rats

Pituitary donor group	No. assay animals†	Ventral prostate mg.
None-(uninjected control)	5	8.8±0.9*
Normal males	6	39.5±4.7
Morphine treated males	5	37.8±4.6

* Standard error.
† Assay animals were 22 day-old, hypophysectomized male rats. Each animal was given a total of 0.75 mg. of dried pituitary material.

TABLE 5

Effect of morphine on pituitary content of FSH in male rats

Pituitary donor group	No. assay animals†	Total dose mg.	Ovaries mg.
None-(uninjected controls)	7	. .	16.5±0.5*
None-(30 IU HCG only)	10	. .	37.8±1.9
Normal males	7	0.1	44.2±2.7
Normal males	7	0.3	96.9±6.5
Morphine treated males	4	0.1	90.0±14

* Standard error.
† Assay animals were 21 day-old intact, female rats. All groups given pituitary material received, in addition, 30 IU of HCG.

trol female glands. Again, as in the earlier assays of female pituitaries, no effect of morphine on the pituitary content of LH was found. In the case of the FSH assays it was found that the control male pituitary glands were far more potent than those of females. Thus, a total dose of 0.3 mg. of pituitary material from male donors produced a greater increase in ovarian weight than was obtained earlier with 3.0 mg. of material from female donors. This indicates that the pituitary glands of male rats of this age normally have more than ten times as much FSH as is contained in the glands of females. As in the case of females, it was also found that morphine injections for two weeks elevated the level of FSH by approximately threefold (Table 5). In the case of the males, therefore, it is possible to state that the increase in number, size, and staining intensity of the centrally placed, PAS-purple gonadotrophs which follows morphine treatment is associated with a marked increase in the pituitary content of FSH. Since no striking alterations in the other pituitary cells were observed this relationship is considered as additional evidence favoring our interpretation that the PAS-purple gonadotrophs are responsible for FSH elaboration and storage (Hildebrand, *et al.*, 1957).

If the above interpretation is correct one may ask why no marked changes in these cells were seen in the glands of the females treated with morphine. It now seems likely that the explanation may lie in the much smaller number of gonadotrophic cells normally present in female glands. Shifts in the relative number of a given cell type that can be observed by microscopic inspection will depend on a number of factors including the initial number of such cells, the individual variability of this number and variability in the distribution pattern of the cell type under study. Since cell counts were not made and only three levels of the glands were studied these considerations would seem adequate to account for the failure to observe any marked effect of morphine on the gonadotrophic cells of the females. Also, it should be pointed out that while no striking changes in the acidophiles were observed, the cytological methods used were not well suited to the study of these cells. It seems quite probable that pituitary functions involving hormones other than gonadotrophins would be affected by such a series of morphine injections.

Finally, while it may have been due to a fortuitous choice of dosage and duration of treatment, it appears that in the present study morphine treatment selectively increased the pituitary store of FSH without affecting LH. The cytological changes which accompanied this hormonal shift involved marked changes in the centrally placed, PAS-purple gonadotrophs. It is concluded, therefore, that these cells are responsible for FSH storage. While not proved by this experiment it seems a reasonable assumption that they are also responsible for the elaboration of this hormone.

Summary

The effect of daily injections of morphine sulfate (60 mg./kg. for a two week period) on the pituitary content of FSH and LH in young adult Holtzman rats was determined by bio-assay. Cytological studies, utilizing the PAS-methyl blue and aldehyde fuchsin methods for gonadotrophs and thyrotrophs, were carried out on other pituitary glands from control and morphine-treated animals. It was found that the morphine treatment led to a persistent diestrous state in approximately one-half of the treated females. In both male and female rats subjected to the morphine injections there was a threefold increase in the pituitary content of FSH but no change in the level of pituitary LH. In the pituitary glands of treated males this increase in the FSH content was accompanied by a marked increase in the number, size and staining intensity of the centrally placed, PAS-purple gonadotrophs. It was concluded that this type of gonadotroph in the rat hypophysis is responsible for FSH elaboration and storage.

REFERENCES

1. Barraclough, C. A., and C. H. Sawyer: Inhibition of the release of pituitary ovulatory hormone in the rat by morphine. *Endocrinology*, 57: 329–337, 1955.
2. Greep, R. O., H. B. Van Dyke, and B. F. Chow: Use of anterior lobe of prostate gland in the assay of metakentrin. *Proc. Soc. Exp. Biol. and Med.*, 46: 644–649, 1941.
3. Hellbaum, A. A., and R. O. Greep: Qualitative changes in the gonadotropic complex of the rat pituitary following the removal of the testes. *Am. J. Anat.*, 67: 287–304, 1940.
4. Hellbaum, A. A., L. G. McArthur, J. P. Campbell and J. C. Finerty: The physiological fractionation of pituitary gonadotropic factors correlated with cytological changes. *Endocrinology*, 68: 144–153, 1961.

5. Hildebrand, J. E., E. G. Rennels, and J. C. Finerty: Gonadotropic cells of rat hypophysis and their relation to hormone production. *Z. Zellforsch.*, 46: 400–411, 1957.
6. Purves, H. D., and W. E. Griesbach: The site of follicle stimulating and luteinizing hormone production in the rat pituitary. *Endocrinology*, 55: 785–793, 1954.
7. Rennels, E. G.: Two tinctorial types of gonadotrophic cells in the rat hypophysis. *Z. Zellforsch*, 45: 467–471, 1957.
8. Rennels, E. G.: The effect of morphine on the pituitary content of FSH and LH in the rat. *Anat. Rec.*, 136: 264, 1960 (Abst.)
9. Steelman, S. L., and F. M. Pohley: Assay of the follicle stimulating hormone based on the augmentation with human chorionic gonadotrophin. *Endocrinology*, 53: 604–616, 1953.

CHEST X-RAY FINDINGS IN EARLY DISSEMINATED *LUPUS ERYTHEMATOSUS**

EDWIN G. TROUTMAN AND DANIEL L. CRESON

Since the recognition in 1872 by Kaposi (5) of lupus erythematosus as a disseminated, systemic disease, the contents of the thorax have been known to harbor various manifestations of this disease and its related collagen disorders. Osler in 1895 (17) cited the preceding literature, which was mostly German, and noted the occurrence of pulmonary lesions. Osler's own cases included instances of pericarditis, pleuritis, and pneumonitis (17, 18). The classic by Libman and Sacks in 1924 mentioned pleural and pericardial involvement (13), and the high incidence of pleuritis and pericarditis was emphasized by Baehr, Klemperer, and Schifrin in their extensive clinical report in 1935 (1). In 1954, Harvey *et al.* published a lengthy review of the literature and an analysis of 105 new cases wherein they noted pericarditis in 45%, pleuritis in 56%, and "lupus pneumonitis" in 22% (8).

Although parenchymal pulmonary infiltrations were long thought to be tuberculous, this was disproved (2, 10, 14, 16), and in 1941 Klemperer, Pollack, and Baehr called attention to the "waxing and waning bronchopneumonia" which was not adequately explained by autopsy findings (12). The next year Rakov and Taylor described the autopsy findings in a single case and focused upon the "atelectasis" which on microscopic examination proved to consist of thickened alveolar septa with a low grade chronic inflammation (20). Later, in 1946, Foldes described similar findings, applied the term "atelectasizing pneumonitis," and hastened to add that some of these changes were to be found in the more normal appearing, non-atelectatic areas (4). Others have confirmed these pathological changes (8, 19, 22).

Changes in the pulmonary vascular tree have been mentioned repeatedly, but not emphasized. The usual vascular change is thickening of the vessel wall in the smaller arteries and arterioles

* Presented to the University of Texas Medical Branch Student Research Forum, April 21, 1961, and to the Texas Chapter, American College of Chest Physicians, April 23, 1961.

and does not include hilar enlargement or an increase in the size of the lumen as in passive congestion (4, 19, 22). Clinical evidence of increased pulmonary pressure has not been generally observed, as in periarteritis nodosa, and in our series only one case had evidence of pulmonary outflow tract enlargement.

Israel in 1953 (9), and then Taylor and Ostrum in 1959 (21), have presented the most detailed studies of the chest X-ray in disseminated lupus erythematosus and the latter authors list pericardial effusion as the earliest sign, followed by pleural effusion, perivascular inflammation, subpleural inflammation, pneumonitis and linear atelectasis, in that order of appearance. At The University of Texas Medical Branch Hospitals we have had numerous occasions to wonder about the manifestations of disseminated lupus erythematosus on the chest X-ray. Our particular interest has been in the changes occurring early in the natural history of this disease and whether or not any evidence could be obtained by chest X-ray to support a clinical suspicion of dissemination in early cases.

Procedure

Cases were selected from the files of the University of Texas Medical Branch Hospitals in which a definite diagnosis of disseminated lupus erythematosus was made upon discharge and in which all chest X-rays were still available. Cases were not included if the data on the chart indicated that such a diagnosis was not clearly justified, if either LE tests or biopsy was not done, or if some other chest or heart disease was present which would obscure early changes that may have been due to disseminated lupus erythematosus.

Of the 82 cases fitting our criteria, only six died before, or soon after discharge; many are still living. Although this low mortality may prejudice the study in favor of less seriously afflicted cases, it enables us to emphasize the chest X-ray findings that occur early in the disseminated phase. Of the total cases, 58 had abnormalities on the chest X-rays which were not obviously due to some complication or other disease; 60 were confirmed by a positive LE test and/or biopsy. The sex incidence was 68 (83%) female and 14 (17%) male; the number of white and colored males was nearly the same, but there were twice as many white females as colored (Table 1).

Approximately 1500 chest X-rays taken on these 82 patients were read. Representative borderline changes were read as unknowns by Robert N. Cooley, M.D., Professor and Chairman of Radiology, John W. Middleton, M.D., Associate Professor of Medicine, and McClure Wilson, M.D., Associate Professor of Radiology. All X-rays were interpreted in accordance with the following outline:

1. Evidence of pericardial and/or myocardial involvement:
   a. generalized cardiac enlargement without loss of chamber identity.

TABLE 1

Age, sex, and race of the 82 cases of disseminated lupus erythematosus in this series

Age	White		Colored		Total
	Male	Female	Male	Female	
0–9	..	..	..	1	1
10–19	..	9	..	3	12
20–29	1	13	3	2	19
30–39	1	11	1	6	19
40–49	1	11	1	7	20
50–59	2	3	..	2	7
Over 60	3	..	..	1	4
Total	8	47	5	22	82

b. a widening of the inferior portion of the cardiac profile.

2. Evidence of pericardial involvement:
   a. straightening of the left cardiac border with obliteration of the normal curves associated with the aorta, pulmonary artery, and left ventricle.
   b. obliteration of a cardiophrenic angle without adjacent pleural changes.
   c. water-bottle contour.
3. Evidence of pleural involvement:
   a. haziness over a lower lung field.
   b. obliteration of a costophrenic sinus.
   c. frank fluid (meniscus sign).
   d. certain cases with a high diaphram (suspected infrapulmonary effusion).
4. Evidence of parenchymal involvement:
   a. linear streaks, or Fleischner's lines, occurring usually in the lower third of the lung and often abutting the pleura at one end or the other.
   b. "lupus pneumonitis," manifested by focal patches of infiltration, ill-defined in both outline and substance, rarely dense or homogeneous, but loosely reticular or granular, and of low contrast (6).
   c. perivascular thickening revealed by an increased diameter of the vascular markings with extension into the peripheral zone (out of keeping with the quality of the X-ray and the age of the patient), usually *not* accompanied by hilar enlargement.

*Illustrative Cases and X-Rays**

Case 1: A 16 year old colored female had skin lesions, negative LE tests, and an X-ray that showed a pneumonic infiltration in the left base, pleuritis over the medial portion of the left dia-

* Clinical bases for the diagnosis of disseminated lupus erythematosus are omitted.

phram, a straightened left cardiac border, and perivascular thickening. Two years later, after the skin lesions had progressed with the interim arrival of the typical "butterfly" lesion, the LE tests were still negative, but the same findings were still present in the chest X-rays (Fig. 1).

Case 2: 37 WF had a skin biopsy showing "collagen disease" and a chest X-ray revealed bilateral patchy pneumonitis and perivascular thickening (Fig. 2). Two months later there was

FIG. 1, Case 1.

FIG. 2, Case 2.

FIG. 3, Case 2.

FIG. 4, Case 2.

frank effusion on the left (Fig. 3). Nearly three years later, the LE tests were positive but most of the X-ray signs had cleared, leaving a somewhat straightened left cardiac border (Fig. 4).

Case 3: 46 CF had a positive LE test and chest X-rays that showed only perivascular thickening (Fig. 5). This was the only patient out of the 30–49 age group with abnormal X-rays that did not have changes in the cardiac profile.

Case 4: 17 WF was admitted for eye surgery and a routine chest X-ray showed perivascular thickening (Fig. 6). Three

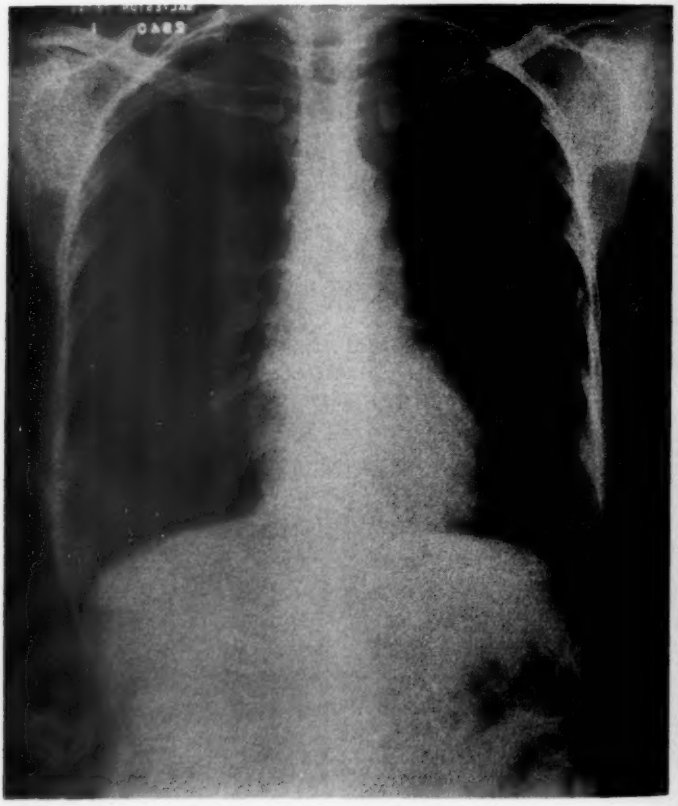


FIG. 5, Case 3.

months later there was clinical evidence of lupus erythematosus and positive LE tests.

Case 5: 15 WF had positive LE tests and X-rays that showed perivascular thickening (Fig. 7). Nearly a year later the LE tests were negative and the perivascular thickening had regressed (Fig. 8).

Case 6: 23 CM had a frank pericardial effusion and pleuritis in the right costophrenic angle (Fig. 9). In the next three years there were three more episodes of frank pericardial effusion and during the last episode positive LE tests were finally obtained.

FIG. 6, Case 4.

Case 7: 46 WF had positive LE tests, ascites, low voltage QRS, and an enlarged heart with inferior widening (Fig. 10). Three years later there was further enlargement of the cardiac profile with a suggestive water-bottle contour, bilateral pleuritis, and pneumonitis (Fig. 11).

Case 8: 30 CF had evidence of pleuritis over the left diaphram and, possibly, some cardiac enlargement (Fig. 12). Two years

FIG. 7. Case 5.

FIG. 8. Case 5.

FIG. 9. Case 6.

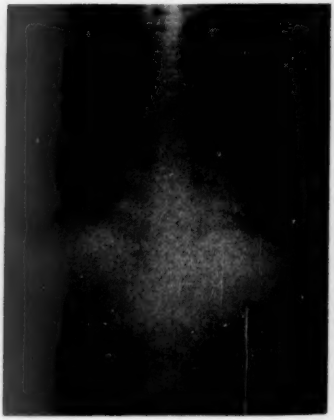


FIG. 10. Case 7.

later lupus erythematosus was suspected and there were definite cardiac and pleural changes (Fig. 13). Four years after the first abnormal X-rays, these changes had largely regressed (Fig. 14), but the LE tests were then positive, and in another year there was suggestive electrocardiographic evidence of pericarditis and some of the cardiac and pleural changes reappeared (Fig. 15).

Case 9: 27 CM did not take a deep breath for the first X-ray which, nevertheless, suggested some haziness in the right base (Fig. 16); the lateral revealed a fluid collection over the right

FIG. 11, Case 7.

FIG. 12, Case 8.

FIG. 13, Case 8.

FIG. 14, Case 8.

diaphram which was elevated (? infrapulmonary effusion) (Fig. 17). One month later there was no evidence of pleural fluid, but the cardiac profile seemed enlarged (Fig. 18). A year and one-half later there was inferior widening, obliteration of the cardiophrenic sinuses, and perivascular thickening (Fig. 19). Skin biopsies revealed "collagen disease."

Case 10: 27 WF had low T waves, perivascular thickening and haziness in the right costophrenic angle (Fig. 20). The lateral

FIG. 15, Case 8.

FIG. 16, Case 9.

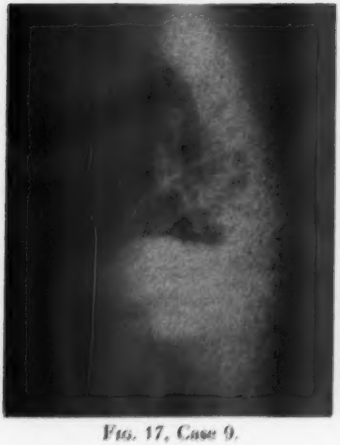


FIG. 17, Case 9.

FIG. 18, Case 9.

revealed fluid behind the right diaphram (Fig. 21) and one year later all changes had disappeared except the perivascular thickening (Fig. 22).

Case 11: 32 WF had diffuse T wave changes, "suspicious" LE tests, a pericardial friction rub, inferior widening, a straight left cardiac border, and perivascular thickening (Fig. 23).

Case 12: 39 WF had a pericardial tap before admission for frank pericardial effusion with electrocardiographic evidence of

FIG. 19, Case 9.

FIG. 20, Case 10.

FIG. 21, Case 10.

FIG. 22, Case 10.

pericarditis. The admission X-ray showed inferior widening, a straight left cardiac border, and bilateral pleuritis (Fig. 24). LE tests and skin biopsy were positive.

Case 13: 38 WF had a straight left cardiac border, obliterated cardiophrenic angles, and bilateral Fleischner's lines (Fig. 25) which were shown more clearly on the lateral just over the posterior portion of the diaphrams (Fig. 26).

Case 14: 46 WF had positive LE tests, a straightened left cardiac border, obliteration of the left cardiophrenic angle, bi-

FIG. 23, Case 11.

FIG. 24, Case 12.

FIG. 25, Case 13.

FIG. 26, Case 13.

lateral pneumonitis, perivascular thickening, and Fleischner's lines in the left base (Fig. 27).

Results

Changes in the cardiac profile were the most frequent—oc-

TABLE 2

Incidence of various groups of X-ray abnormalities in the chest in patients with disseminated lupus erythematosus according to age distribution. The frequency distribution in each of the three systems follows the general age distribution; the peak age incidence is one to two decades later than in most other series published. In the two age groups, 30–39 and 40–49, there is only one case having an abnormal X-ray that did not have changes in the cardiac profile (Case 3).

Age	Total number	Number with positive X-rays	Number with particular signs		
			Heart	Lungs	Pleura
0–9	1	1	1	1	1
10–19	12	6	2	5	3
20–29	19	10	8	8	7
30–39	19	16	16	7	8
40–49	20	14	13	10	10
50–59	7	7	5	6	3
Over 60	4	4	2	3	3
Totals	82	58	47	40	35
Per cent of total cases		71%	57%	49%	43%
Per cent of those with positive X-rays			80%	69%	60%

FIG. 27, Case 14.

curring in 80% of those cases with abnormal X-rays and in 57% of the total series (Table 2). Although the least common of the cardiac changes was the water-bottle contour indicative of frank pericardial effusion, other changes that Taylor and Ostrum ascribed to pericarditis without frank effusion (21) were very frequent (Table 3). By far the most interesting of the cardiac

TABLE 3

Incidence of changes in the cardiac profile in the 47 patients which had abnormalities of the cardiac contour

	Number of cases	Incidence of findings		
		Of all heart changes	Of all X-ray changes	Of total series
Obliterated left cardiophrenic angle	33	70%	57%	40%
Inferior widening of the cardiac profile	30	64%	53%	37%
Straight left cardiac border	27	58%	47%	33%
Generalized enlargement	20	43%	34%	24%
Water-bottle contour	5	11%	9%	6%

changes was the alteration in the contour of the left cardiac border. A straightening of this border which obliterated the normal concavities separating the contours of the aorta, pulmonary conus, and left ventricle was found in 58% of those cases having any change in the cardiac profile. This abnormality becomes more apparent when accompanied by an obliteration of the left cardiophrenic angle. Cases 11 and 12 are examples of these changes concurrent with other evidence of pericarditis. These observations support the findings of Taylor and Ostrum (21). Less specific are the findings of generalized cardiac enlargement and inferior widening. However, each of these assumes considerably more importance when dynamic rather than static. The generalized enlargement could be either minimal pericardial effusion or dilatation due to myocarditis; the inferior widening is most likely caused by a small collection of pericardial fluid. Inferior widening is not likely due to hypertension, as there was no correlation between this sign and blood pressure readings over 140/90. That generalized enlargement of the cardiac profile and

inferior widening can represent pericardial effusion is certain since they often precede or succeed overt evidence of pericardial fluid and/or pericarditis.

Perivascular thickening was the most common parenchymal change—having been found in 65% of those having any parenchymal alterations (Table 4). This sign is probably very signifi-

TABLE 4

Incidence of changes in the pulmonary parenchyma in the 40 patients which had abnormalities in the lung fields

	Number of cases	Incidence of findings		
		Of all lung changes	Of all X-ray changes	Of total series
Perivascular thickening	26	65%	45%	32%
Linear atelectasis	14	35%	24%	17%
"Lupus pneumonitis"	11	27%	19%	13%

cant, albeit very hard to evaluate, particularly in those individuals under 20 years of age and/or where the quality of the X-ray is "soft." Therefore, in evaluating perivascular thickening objectively, these two factors have to be considered constantly. The two large reproductions (Figs. 5 and 6) show perivascular thickening of the smaller vessels and extension of the vascular markings into the peripheral zones. Although much contrast and detail has been lost due to the inferior resolution of photographic film compared to radiographic film, they still give a reasonable representation of this frequent and important sign. Perivascular thickening is sometimes difficult to distinguish from finely scattered "lupus pneumonitis" and the two may occur simultaneously (Fig. 2). The two other important parenchymal changes, linear atelectasis (Fleischner's lines) and "lupus pneumonitis," occurred less frequently than perivascular thickening (Table 4).

Pleural changes were the least frequent in the total series and probably the least indicative of disseminated lupus erythematosus. Although more than half of those with pleural findings had frank fluid, this was often minimal in amount and preceded by evidence of pleuritis for variable lengths of time before free fluid was manifest. Table 5 summarizes the pleural changes.

TABLE 5

Incidence of changes in the pleura in the 35 patients which had abnormalities in the pleurae

	Number of cases	Incidence of findings		
		Of all pleural changes	Of all X-ray changes	Of total series
Frank fluid (meniscus)	19	54%	33%	23%
Obliterated angle	15	43%	26%	18%
Haziness over base	12	34%	21%	15%
High diaphram (? infra-pulmonary effusion)	9	26%	16%	11%

Correlation of these various findings is interesting and revealing. A table was constructed to match the incidence of any one sign to the incidence of all other signs. From this table each combination was selected which represented 45% or more of those patients having each of the two findings being compared. For instance, 19 patients had both a straightened left cardiac border and inferior widening of the cardiac profile. These 19 patients represented 70% of all those having a straightened left cardiac border and 61% of all those having inferior widening of the cardiac profile. Similarly, each of the six combinations in Table 6 have a correlation of greater than 45% for each of the signs in each combination. These six combinations represent four separate signs: a straight left cardiac border, an obliterated left cardiophrenic angle*, inferior widening of the cardiac profile, and perivascular thickening.

With these six combinations of four signs, 33 patients, representing 57% of those with any X-ray abnormality and 40% of the total series, can be identified without regard to other radiographic abnormalities on the chest X-ray. These 31 patients had 91 instances of combinations of two of these four signs, indicating that most patients had more than one combination.

Cytoid bodies in the retinae are probably the most specific of the physical signs of disseminated lupus erythematosus. Although it has not been customary to examine all suspected cases carefully for cytoid bodies, it is the opinion of the authors that this procedure could be an important adjunct to the evaluation of

* The left cardiophrenic angle was obliterated in every case where the right angle was also obliterated.

TABLE 6

Number of cases and incidence of each of the six combinations in which each sign represented more than 45% of all the cases exhibiting that particular sign

Combination	Number of cases	Incidence of findings	
		Of all X-ray changes	Of total series
Straight left border and inferior widening	19	33%	23%
Obliterated left cardiophrenic angle and inferior widening	16	28%	20%
Obliterated left cardiophrenic angle and straight left border	15	26%	18%
Straight left border and perivascular thickening	14	24%	17%
Inferior widening and perivascular thickening	14	24%	17%
Obliterated left cardiophrenic angle and perivascular thickening	13	22%	16%

Total number of combinations: 91 instances in 33 patients representing 57% of those having any type of X-ray change and 40% of the total series.

disseminated lupus erythematosus, especially when the LE tests are negative.

Summary

1. Neither diagnostic nor pathognomonic findings were observed. However, pleural, cardiac, and parenchymal changes are common in disseminated lupus erythematosus.

2. Any combination of two of four signs can be taken as major radiographic evidence to support a clinical suspicion of disseminated lupus erythematosus. These four signs are:

a. a straight left cardiac border
b. an obliterated left cardiophrenic angle
c. inferior widening of the cardiac profile
d. perivascular thickening.

3. The remaining changes can be considered as minor supporting signs. They are:

a. generalized cardiac enlargement
b. frank pericardial effusion
c. frank pleural effusion
d. pleuritis

e. linear atelectasis (Fleischner's lines)

f. "lupus pneumonitis."

4. These major and minor criteria should be evaluated in the light of the patient's clinical history and physical findings, and do not represent radiographic signs of pathognomonic significance. If, after careful clinical evaluation, there is a suspicion of lupus erythematosus, these signs may help in deciding whether or not disseminated disease is present, and may occasionally be the earliest indication of disseminated involvement.

REFERENCES

1. Baehr, G., P. Klemperer, and A. Schrifrin: A diffuse disease of the peripheral circulation (usually associated with lupus erythematosus and endocarditis). *Tr. A. Am. Phys.*, 50: 139–155, 1935.
2. Bille, B. S. V.: Lupus erythematosus disseminatus with and without skin eruptions. *Acta med. scand.*, 140: 280–289, 1951.
3. Fleischner, F., A. O. Hampton, and B. Castleman: Linear shadows in the lung (interlobar pleuritis, atelectasis and healed infarction). *Amer. J. Roentg.*, 46: 610–618, 1941.
4. Foldes, J.: Acute systemic lupus erythematosus. *Amer. J. Clin. Path.* 16: 160–173, 1946.
5. Gahan, E.: Original description of lupus erythematosus. *Arch. derm. and syph.*, 66: 458–459, 1952.
6. Gould, D. M., and M. L. Daves: Roentgenologic findings in systemic lupus erythematosus, an analysis of 100 cases. *J. Chron. Dis.*, 2: 136–145, 1955.
7. Gross, L.: The cardiac lesions of Libman-Sacks disease with a consideration of its relationship to acute diffuse lupus erythematosus. *Amer. J. Path.*, 16: 375–407, 1940.
8. Harvey, A. McG., L. E. Shulman, P. A. Tumulty, C. L. Conley, and E. H. Schoenrich: Systemic lupus erythematosus: review of the literature and clinical analysis of 138 cases. *Medicine*, 33: 291–437, 1954.
9. Israel, H. L.: The pulmonary manifestations of disseminated lupus erythematosus. *Amer. J. Med. Sci.*, 226: 387–392, 1953.
10. Keil, H.: Relationship between lupus erythematosus and tuberculosis, a critical review based on observations at necropsy. *Arch. derm. and syph.*, 28: 765–779, 1933.
11. Klemperer, P.: The pathogenesis of lupus erythematosus and allied conditions. *Ann. Int. Med.*, 28: 1–11, 1948.
12. Klemperer, P., A. D. Pollack, and G. Baehr: Pathology of disseminated lupus erythematosus. *Arch. path.*, 32: 569–631, 1941.
13. Libman, E., and B. Sacks: A hitherto undescribed form of valvular and mural endocarditis. *Arch. Int. Med.*, 33: 701–737, 1924.
14. MacLeod, J. M. H.: Lupus erythematosus, some variations on its etiology. *Arch. derm. and syph.*, 9: 1–12, 1924.
15. Maumenee, A. E.: Retinal lesions and lupus erythematosus. *Amer. J. Ophth.*, 23: 971–981, 1940.

16. Mook, W. H., R. S. Weiss, and L. Bromberg: Lupus erythematosus disseminatus. *Arch. derm. and syph.*, 24: 786–829, 1931.

17. Osler, W.: On the visceral complications of erythema exudativum multiforme. *Amer. J. Med. Sci.*, 110: 629–646, 1895.

18. ————: On the visceral manifestations of the erythema group of skin diseases. *Amer. J. Med. Sci.*, 127: 1–23, 1904.

19. Purnell, D. C., H. Bagenstoss, and A. M. Olsen: Pulmonary lesions in disseminated lupus erythematosus. *Ann. Int. Med.*, 42: 619–628, 1955.

20. Rakov, H. L., and J. S. Taylor: Acute disseminated lupus erythematosus without cutaneous manifestations and with heretofore undescribed pulmonary lesions. *Arch. Int. Med.*, 70: 88–100, 1942.

21. Taylor, T. L., and H. Ostrum: The roentgenologic evaluation of systemic lupus erythematosus. *Amer. J. Roentg.*, 82: 95–107, 1959.

22. Teilum, G.: Pathogenetic studies on lupus erythematosus disseminatus and related diseases. *Acta med. scand.*, 123: 126–142, 1946.

23. Tumulty, P. A., and A. McG. Harvey: The clinical course of disseminated lupus erythematosus, an evaluation of Osler's contributions. *Bull. J. Hopkins Hosp.*, 85: 47–73, 1949.

AN INVESTIGATION OF INTRINSIC SITES OF DEMINERALIZATION IN BONE: A MICRORADIOGRAPHIC STUDY[a]

GEORGE P. VOSE[b]

Changes in the mineral content of bone may occur in numerous pathological and nutritional disturbances—often to such a marked extent that it is apparent upon visual inspection of the radiograph. In certain metabolic diseases, such as osteoporosis and osteomalacia, the decreased mineral content is reflected by a high degree of bone radiolucency. In osteopetrotic bone, on the other hand, the increased mass of absorbing material results in a marked X-ray attenuation by the bone. Chronic nutritional deficiencies may also be reflected by changes in bone mineral content.

While gross changes in mineralization can be diagnosed by visual inspection of the radiograph, the method lacks objectivity. Small changes of only a few percentage values, however, can be determined without biopsy by densitometric measurements or analytical determinations of emulsion silver. Mack (8, 9, 10) was an early investigator in the field of bone density measurements, and similar *in vivo* techniques for the evaluation of bone density from radiographs have been used by other investigators (1, 2, 6, 7, 11, 13, 14, 15, 16, 22, 23). Methods have been described for measuring bone density by chemical quantitative analysis of emulsion silver, (17) and by use of a collimated polychromatic X-ray beam (12, 18, 19) and a gamma source utilizing Iridium-192 (6). In addition to these *in vivo* techniques we have used monochromatic K-capture radiation of Iron-55 to measure the mineral content of semimicro amounts of bone in solution (20).

Unfortunately the intrinsic sites of mineral loss and gain are masked upon the radiograph by the complexly superimposed images of innumerable structures within the bone. The optical

[a] Supported in part by Grant No. A-2641 from the National Institute of Arthritis and Metabolic Diseases, The National Institutes of Health, U. S. Public Health Service. Submitted for publication March 20, 1961.

[b] From the Nelda Childers Stark Laboratory for Human Nutrition Research, Texas Woman's University, Denton, Texas.

density of the radiographic image is representative of the mass of absorbing material without regard to its location within the bone, and since bone is a nonhomogeneous substance with variable concentrations of mineral and matrix components, neither the physical density of the bone nor its radiographic density is directly related to its mineral content. Our investigations of senile osteoporotic bone, for example, have shown that the atrophied cortex in osteoporosis has a high mineral concentration in spite of its overall thinness (21). Hence the mineral concentration in small areas of a bone may be abnormally high even though the mineral content of the entire bone may be low on a weight-per cent basis.

Although the microscopic sites of initial changes in bone mineralization are not clearly known, demineralization of cortical bone appears to follow one of two patterns: (a) as a generalized, evenly distributed condition in Haversian or extra-Haversian bone, and (b) in localized "pockets" in both Haversian and extra-Haversian bone. It must be pointed out that osteoporosis is an atrophic condition, and the accompanying decrease in bone mass is not "demineralization" in its true sense.

In the search for the intrinsic sites of initial occurrence of demineralization under various pathological conditions it is of particular interest to study a bone affection known as "feathering" which has been described by Frost (4, 5). The condition is characterized by the presence of an irregular distribution of mineral in various quadrants of the Haversian systems, and occasionally in extra-Haversian bone. The affection is readily discernible in basic fuchsin stained sections of undecalcified bone which is normally impervious to the stain although it is drawn into the lacunae, canaliculae, and the Haversian and Volkman canals. Incompletely mineralized bone, on the other hand, has an affinity to fuchsin stain which increases in accordance with the degree of demineralization. Feathered bone appears to be incompletely mineralized since it stains diffusely with fuchsin, while adjacent, normally mineralized bone absorbs but little of the stain (4, 5).

Feathered bone has been studied in the present investigation using both basic fuchsin stained sections and microradiographs in order to determine quantitatively the degree of demineralization in feathered osteones.

Materials and Methods

Approximately 20 sections of undecalcified bone removed from the femur cortex of 12 individuals were ground to a thickness of 20 to 35 microns and microradiographed at 4 KV, 2 MA, and 6 minutes using a Philips CMR-5 Contact Microradiographic Unit and Type 649-0 film. Ilford formula 36 developer was used with standard processing techniques.

Following microradiography the sections were stained for 48 hours in 1% basic fuchsin in 40% ethanol using Frost's technique (3). Next, the sections were ground lightly between glasses covered with No. 400A silicon carbide abrasive paper to remove obscuring surface stain. The stained sections were viewed in an optical microscope to determine the sites of feathering, as indicated by diffuse stain retention, prior to densitometric analysis.

Densitometric Determinations. In measuring the X-ray absorbency of feathered bone from the microradiographs, a step wedge was constructed of aluminized Mylar with each step having a thickness of 1.8 mg/cm^2. The overall length of the wedge was 2.5 mm. with a maximum thickness of 135 microns. This was microradiographed using the same exposure and processing conditions that were used with the bone section. As described in an earlier publication, (10) the inherent nonlinearity of the sensimetric curve will result in a nonlinear displacement of the individual steps when the wedge is scanned in a microphotometer, and nonlinearity is also present in the bone scan. The instrument used in the present work is capable of automatic correction for film nonlinearity so that the area under the bone scan is in direct proportion to x-ray absorbance by the bone section. With a scanning aperture of 0.25 mm. and a film velocity of 0.1 mm/min, the resolving power of the instrument is approximately ten microns.

Normal Haversian bone and Haversian bone which had areas of apparent feathering as indicated by its affinity for basic fuchsin, were scanned in the modified Knorr-Albers micro-densitometer. Immediately thereafter the aluminized Mylar step wedge was scanned at the same exciter voltage. Since the step representation was brought to linearity, a direct interpolation could be made between the recorder deflection of the feathered bone image and that of the step wedge. Final readings are in terms of the x-ray aluminum equivalency in milligrams per micron of bone thickness.

Observations

Of the 12 femurs examined, nine showed evidence of varying degrees of feathering as indicated by fuchsin permeability. Microradiographs made prior to the staining process indicated that the feathered areas had a conspicuously lower radiopacity than adjacent nonfeathered extra-Haversian bone. Hence microradiography can provide a means for diagnosing bone feathering, and it also makes quantitative analysis possible through densitometric evaluation of the films.

The lower photograph in Figure 1 illustrates that newly formed

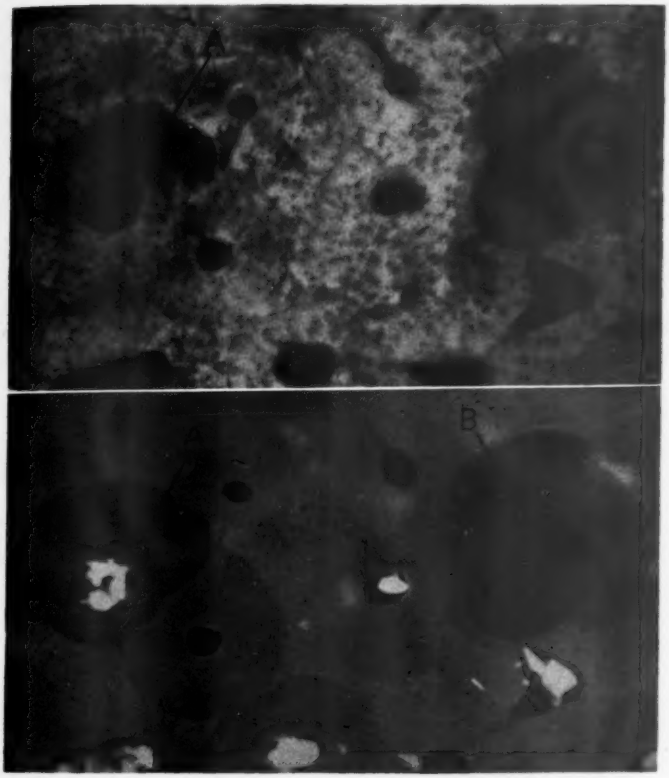


FIG. 1. Photomicrograph (upper) of fuschin stained cortical bone showing feathered areas (A and B), and microradiograph (lower) of same section.

Haversian bone is less dense than the surrounding extra-Haversian bone as shown by its lower X-ray absorbency. The figure also indicates that feathered Haversian bone is less dense than nonfeathered Haversian bones (sites A and B). For comparative purposes a photomicrograph of the same section is shown after being stained with basic fuchsin, and the same feathered areas are indicated by the dark, diffused patches as a result of absorbed stain. The areas are somewhat more poorly defined in the stained section than in the microradiograph.

Since the X-ray absorption at 4 KV is, for all practical pur-

poses, accountable only to the mineral fraction of bone, then the darker X-ray image of feathered bone indicates a decreased mineral content. Table 1 gives the average aluminum equivalency

TABLE 1

Aluminum equivalency per micron of bone thickness of feathered and nonfeathered Haversian bone, and per cent mineral deficiency in feathered bone

Bone number	Aluminum equivalency per micron of bone (mg/cm^2)		Mineral deficiency in feathered Haversian bone (per cent)
	Nonfeathered Haversian bone	Feathered Haversian bone	
1	.520	.371	28.6
2	.423	.395	6.6
3	.440	.416	5.4
4	.460	.226	50.9
5	.414	.326	21.2
6	.424	.346	18.4
7	.487	.359	13.6
8	.426	.249	41.5
9	.451	.212	53.0
Average	.450	.322	26.6

for both feathered and nonfeathered Haversian bone, and indicates that feathering may account for mineral losses of as much as 53 per cent in certain quadrants of the Haversian system. The average mineral loss in the feathered areas of the nine bones examined was 26.6 per cent, with a total variation between 5.4 and 53.0 per cent according to the severity of the affection.

The etiology of feathered bone is not clearly understood. It may represent either a partial demineralization of originally normal bone, or a delay in mineralization of forming bone. Either case will result in an effective decrease in total bone mineral. Three of the bones in the present study—bones 1–3 of Table 1—exhibited osteoporotic symptoms, but no significant differences were noted in the extent of feathered Haversian systems in osteoporotic and normal femurs. Further work will be required, using both pathologic and normal bone material of varied age groups, to determine the relationship of feathering to gross changes in bone density.

Summary

In an investigation of the intrinsic sites of mineral loss in bone, an affection known as "feathering" was studied microradiographically. Densitometric measurements of partially or wholly feathered Haversian systems indicated that the mineral content of feathered bone may be as low as 53 per cent that of normal Haversian bone.

Acknowledgments

The author is indebted to Mr. Michael Hutcheson for valuable technical assistance, and to Dr. Harold M. Frost, Henry Ford Hospital, for his constructive advice.

BIBLIOGRAPHY

1. Balz, G., R. Birkner, and J. M. Schmitt-Rohde: The calcipenis osteopathies and their diagnosis by means of a special x-ray method. *Arztliche Wochenschrift*, 12: 209–213, 233–237, 1957.
2. Facto, L. A., J. Kastelic, and D. V. Catron: Estimating ash content of bone using standardized radiographs. *Journal of the Biological Photographic Association*, 24: 13–21, 1956.
3. Frost, H. M.: Preparation of thin undecalcified bone sections by rapid manual method. *Stain Technology*, 33: 273–276, 1958.
4. Frost, H. M.: New bone affection: Feathering. *Journal of Bone and Joint Surgery*, 42A: 447–456, 1960.
5. Frost, H. M.: *In vivo* impermeability of feathered bone to tetracyclines. *Henry Ford Hospital Bulletin*, 8: 225–227, 1960.
6. Gershon-Cohen, J., N. H. Cherry, and M. Boehnke: Bone density studies with a gamma gage. *Radiation Research*, 8: 509–515, 1958.
7. Henny, G. C.: Roentgenographic estimation of the mineral content of bone. *Radiology*, 54: 202–209, 1950.
8. Mack, P. B., A. T. O'Brien, J. M. Smith, and A. W. Bauman: A method for estimating the degree of mineralization of bones from tracings of roentgenograms. *Science*, 89: 467, 1939.
9. Mack, P. B., W. N. Brown, Jr., and H. D. Trapp: The quantitative evaluation of bone density. *The American Journal of Roentgenology and Radium Therapy*, 61: 808–825, 1949.
10. Mack, P. B., G. P. Vose, and J. D. Nelson: New development in equipment for the roentgenographic measurement of bone density. *The American Journal of Roentgenology, Radium Therapy and Nuclear Medicine*, 82: 303–310, 1959.
11. Marchal, M.: Bone micro-densigrams in man and the ponderal evaluation of bone calcium by x-rays. *Compte-Rendus de l'Academie des Sciences*, 226: 526–528, 1948.
12. Mayer, E. H., H. G. Trostle, E. Ackerman, H. Schraer, and O. D. Sittler: A scintillation counter technique for the x-ray determination of bone mineral content. *Radiation Research*, 13: 156–167, 1960.

13. Omnell, K. A.: Quantitative roentgenologic studies on changes in mineral content of bone *in vivo*. *Acta Radiologica*, Supplementum 148, 1957.

14. Reich, S. B., J. Levitin, and L. R. Felton: A roentgen method of evaluating density of bone. *American Journal of Roentgenology, Radium Therapy, and Nuclear Medicine*, 79: 705–708, 1958.

15. Rowland, R. E., J. Jowsey, and J. H. Marshall: Microscopic metabolism of calcium in bone. III. Microradiographic measurements of mineral density. *Radiation Research*, 10: 234–242, 1959.

16. Schraer, H., R. Schraer, H. G. Trostle, and A. D'Alfonso: The validity of measuring bone density from roentgenograms by means of a bone density computing apparatus. *Archives of Biochemistry and Biophysics*, 83: 486–500, 1959.

17. Virtama, P.: Determination of the mineral content of human finger bones by silver analysis of roentgenograms. *Acta Anatomica*, Supplementum 29, 1957.

18. Vose, G. P.: X-ray transmission factor in estimating bone density. *Radiology*, 71: 96–101, 1958.

19. Vose, G. P.: Quantitative determination of osseous and soft fractions of bone by x-ray absorption. *Laboratory Investigations*, 8: 1540–1546, 1959.

20. Vose, G. P.: Determination of small amounts of mineral in bone, cartilage, and muscle by x-ray absorption. *Microchemical Journal*, 4: 537–549, 1960.

21. Vose, G. P., B. J. Stover, and P. B. Mack: Quantitative bone strength measurements in senile osteoporosis. *Journal of Gerontology*, 4: 537–549, 1961.

22. Wallgren, G., and K. Holmstrand: Technical considerations on the quantitative microradiography of bone. *Experimental Cell Research*, 12: 188–191, 1956.

23. Williams, D. E., and A. Samson: Bone density of East Indian and American students. *Journal of the American Dietetic Association*, 36: 462–466, 1960.

STABILIZATION OF POLIOVIRUS BY CATIONS*

CRAIG WALLIS AND JOSEPH L. MELNICK

Viruses are rapidly inactivated by heat (28, 1, 12, 29, 13), although partial stabilization has been reported by addition of broth (1, 19), serum (19, 14), milk and cream (9, 10, 2), and L-cystine (20). Thirty years ago Burnet and McKie (5) reported that monovalent cations enhanced the inactivation of bacteriophage by heat and that the addition of low concentrations of divalent cations partially prevented this inactivation. For example, in the presence of 0.0005 M NaCl, heating for one hour at 60° C. inactivated 70% of the bacteriophage. Raising the NaCl concentration to 0.15 M increased the inactivation to over 99% under the same thermal conditions. When $CaCl_2$ (or $MgCl_2$ or $BaCl_2$) was added at 0.002 to 0.01 M, it protected against the thermal-enhancing effect of the 0.15 M NaCl. However, at a higher concentration than 0.15 M the divalent cations themselves enhanced the inactivation of the bacteriophage at 60° C. Lark and Adams (12) reported stabilizing effects on bacteriophage of Mg ions at 0.000001 M and of Na ions 0.1 M. They suggested that a dissociable complex was formed by the ions and vulnerable sites on the phage particles, the complex being more heat resistant than the cation-free site. Adams (1) showed that bacteriophage could be stabilized at 37° C. for 24–48 hours with 0.001 M concentrations of many cations (Ba, Ca, Sr, Mg, Mn, Co, Ni, Zn, Cd and Cu). Allisbaugh (3) reported that hypertonic NaCl, KCl, or NH_4Cl in high concentration (5 to 40%) stabilized smallpox virus to the extent that the virus remains viable about twice as long at 5° C. as virus suspended in glycerinated diluent.

When present in high concentrations, monovalent and divalent cations appear to stabilize infectious ribonucleic acid (RNA) of certain viruses against heat. Norman and Veomett (18) reported that raising the molarity of NaCl from 0.12 M to 1.2 M resulted in a twofold increase of survival at 60° C. of RNA from poliovirus. When seeded on susceptible cells in the presence of high concentrations of salt (1 M NaCl or 2 M $MgSO_4$) the in-

* From the Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas.

fectivity of polio RNA is increased (11, 7). However, this increase appears to be based on increased cellular permeability to RNA rather than on thermal stabilization of the virus, for after a few minutes of contact with susceptible cells, the salts must be washed from them; otherwise the cytotoxic action of the salts become irreversible. At the time of our work, no stabilizing agent had been described to our knowledge that would completely protect a virus, at any given temperature from 0° to 50° C., when maintained at that temperature for a sufficient period of time during which control virus without stabilizer would become at least 99 per cent inactivated.

This report is concerned with cationic protection, whereby virtually the entire population of infectious poliovirus particles is stabilized at 50° C. for 1–2 hours, at 37° C. for at least 3 days, at room temperature (25°–28° C.) for at least 25 days, and for much longer periods at lower temperatures.

Materials and Methods

Monkey kidney (MK) cells.—Kidneys from immature rhesus monkeys were trypsinized, grown, and maintained in Melnick's medium as described in detail elsewhere (15, 24).

Viruses.—Virulent strains were plaque purified lines of poliovirus type 1 (Mahoney), type 2 (MEF_1) and type 3 (P24). Attenuated strains were those used in the oral poliovaccine (Sabin), and used after one MK passage at this laboratory. They were grown in MK cells maintained in Melnick's medium without serum. Virus harvests were centrifuged to remove cell debris.

Virus assays.—All virus titrations were carried out by determination of plaque-forming units (PFU), using the bottle technique (8). Medium for overlay consisted of Earle's salt solution, 0.1% skim milk, 0.4% $NaHCO_3$, 0.0017% neutral red, in a final concentration of 1.5% agar. $MgCl_2$ at a concentration of 25 mM was incorporated into the agar to expedite plaque counting (25).

Heating Methods.—Virus containing tissue culture fluids were diluted tenfold in 0.5% lactalbumin enzymatic hydrolsate in Earle's salt solution (M-E), and then mixed with an equal volume of the salt or diluent under test. Before it was mixed with virus, the salt was at twice the final concentration desired.

Aliquots to be heated were delivered directly to the bottom of tubes in 0.3 ml amounts, and the top of each tube was flamed thoroughly to sterilize at least one inch of the uppermost surface of the tube. (All test tubes used were uniform in size and thickness, measuring 13 × 100 mm, with an 0.8 mm wall.) The tube was then rubber stoppered and plunged into the water bath, at a depth sufficient to cover the tube to within ¼-inch of the stopper. At the end of the heating period, the tubes were quickly transferred into an ice-water bath. Additional methods are described in the text.

Results

Stabilization of Poliovirus by Cations at Different Temperatures.—The three attenuated strains used in the vaccine and the three virulent prototype strains could all be protected from thermal inactivation. Since, as shown in Table 1, the attenuated and

TABLE 1

Log_{10} virus inactivated at 50° C.

Final concentration of salt	Heating period in hours	Attenuated poliovirus			Virulent poliovirus		
		Type 1 1×10^7	Type 2 3×10^6	Type 3 1×10^7	Type 1 3×10^7	Type 2 1×10^6	Type 3 3×10^7
		(Titers of unheated virus stocks are given in the line above as PFU/ml)					
1 M	1	0.0	0.0	0.0	0.0	0.0	0.0
$MgCl_2$	4	0.4	0.3	1.4	0.4	1.1	0.8
	16	1.2	1.3	3.3	1.5	3.1	3.2
1 M	1	0.0	0.0	..	..	..	..
$CaCl_2$	4	0.7	0.7	1.6	0.5	1.1	1.0
	16	1.6	1.5	3.8	1.8	3.5	3.5
2 M	1	0.4	..	..	0.2	..	..
NaCl	4	1.8	2.8	3.0	2.0	1.5	1.2
	16	>4.0	>4.0	4.0	3.0	>4.0	>4.0
1.0 M $ZnSO_4$	1	0.8	1.5	1.0	2.0	1.1	2.0
0.5 M $CuSO_4$	1	>3.0	>3.0	>3.0	>3.0	>3.0	>3.0
1.0 M $MnSO_4$	1	0.6	..	..	0.5	..	..
1.0 M $CoCl_2$	1	0.1	..	..	..	..	..
	4	0.4	..	..	..	..	..
H_2O	1	>4.0	>4.0	>4.0	2.5	3.5	1.4
H_2O	4	>4.0	>4.0	>4.0	>4.0	>4.0	3.0

virulent strains behaved similarly with the various cations used, only the data for type 1 attenuated strain will be cited in further detail throughout this report.

Fig. 1 illustrates heating experiments at 50° C. with 10^7 PFU/ml of type 1 attenuated virus with 1 M Mg^{++}, CA^{++}, Zn^{++}, Mn^{++}, Co^{++}, K^+, 2 M Na^+ and 0.5 M Cu^{++}. At the molarity used, certain salts caused pH changes with the virus stock sometimes falling to pH 3.8. Since Mg^{++} was used as an index of the sparing effect of cations upon the virus, aliquots of the magnesium-virus mixtures were adjusted with HCl to pH

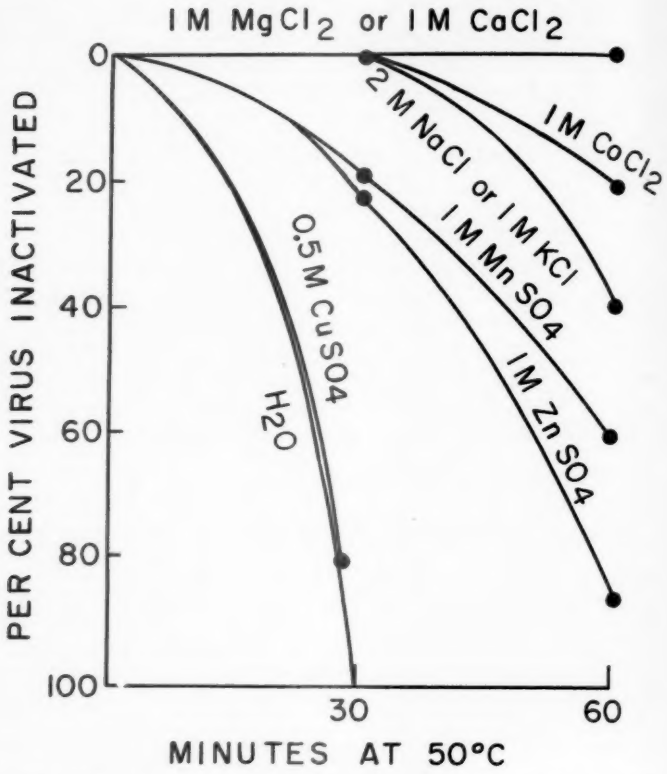


FIG. 1. Stabilization at 50° C. of attenuated type 1 poliovirus, 10^7 PFU/ml. heated in final salt concentrations shown in figure. $MgSO_4$ provided the same degree of stabilization as $MgCl_2$.

levels of 3.8, 4.5, 5.5, 5.7, and 7.0. At all these pH levels, Mg^{++} (added as $MgCl_2$ or $MgSo_4$) afforded complete protection to the virus for 1 hour at 50° C. The only cation that failed to show significant sparing effects was Cu^{++}.

With Mg^{++}, Ca^{++}, K^+, and Na^+ the virus-salt mixtures at the concentrations indicated in the above paragraph could be seeded on MK cells after a tenfold dilution was made, since this brought them beyond the cytotoxic levels of the salts. Because Cu^{++}, Zn^{++}, Co^{++}, and Mn^{++} were more toxic to the cells,

virus-salt mixtures with these cations had to be diluted one-thousandfold before the mixture could be assayed for virus.

When mixed with an equal volume of virus contained in M-E medium, 2 M Ca^{++}, Zn^{++}, or Mn^{++} caused a precipitate to form. Our tests for virus in the supernatant fluids or in the resuspended precipitates showed that the major portion of the infective virus population was sedimented with the precipitate. Grossowicz et al (6) have already reported the use of Ca^{++}, Co^{++}, Mn^{++}, and Ba^{++} for the concentration of polioviruses. However, we failed to observe a precipitate when 2 M Mg^{++} was mixed with the virus, and even centrifugation of the $MgCl_2$-virus mixture failed to sediment any virus.

Fig. 2 shows the results of an experiment where the three types

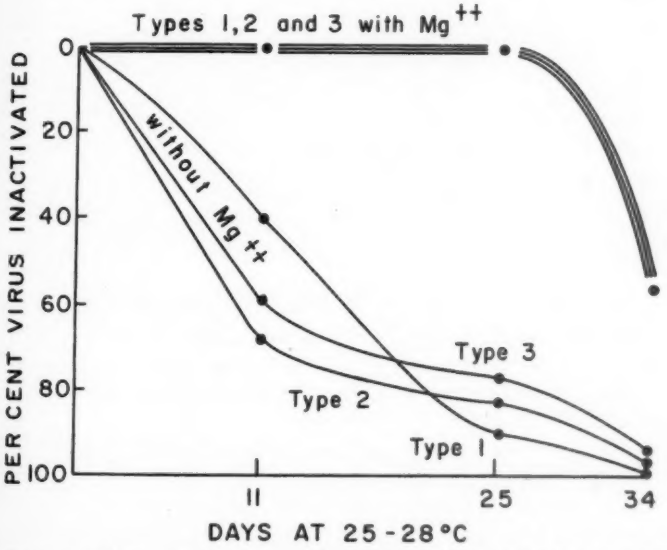


FIG. 2. Stabilization of attenuated polioviruses by molar Mg^{++} at room temperature.

of attenuated poliovirus (types 1 and 3 at $10^{7.0}$ PFU/ml, and type 2 at $10^{6.5}$ PFU/ml) in a final concentration of 1 M Mg^{++} were stored at room temperature, 25–28° C. The viruses held in the $MgCl_2$ solution showed no loss in infectivity even after 25 days of storage, whereas the controls without added $MgCl_2$ lost between

40 and 70 per cent of activity by the 11th day, and between 75 and 90 per cent by the 25th day. By the 34th day the Mg-virus aliquots showed 50 per cent inactivation as compared to 97 and 99 per cent in the control. Stabilization of the virus by M Mg^{++} at 37° C. is evidenced by the data shown in Fig. 3.

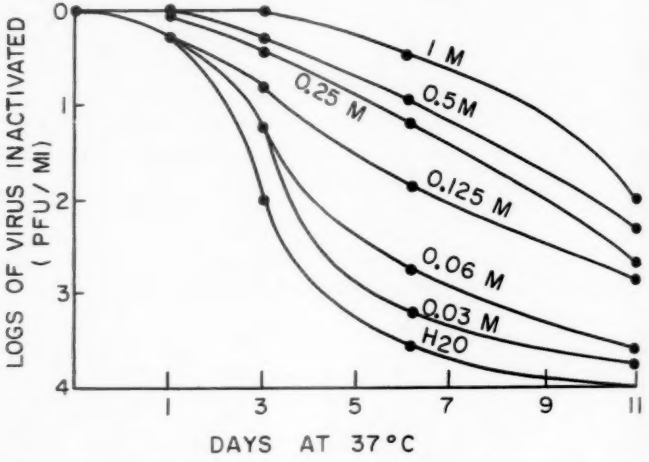


FIG. 3. Effect of Mg^{++} concentration on stability of attenuated type 1 poliovirus at 37° C.

A duplicate series placed at 4° C., and tested after 90 days of storage, showed no loss in virus infectivity in the Mg-virus samples. However, types 1, 2, and 3 attenuated viruses stored without added Mg ions lost over 75 per cent of their infectivity (see Fig. 4).

The Effect of Different Concentrations of Mg^{++}.—Results of a typical experiment with virus at 10^7 PFU/ml but with varying concentrations of $MgCl_2$ (or $MgSO_4$) held at 50° C. for 60 minutes are shown in Fig. 5. The optimum sparing effect was reached at 0.5 M. With a twofold reduction of Mg^{++} to 0.25 M, there was a tenfold loss of infectivity. With a further serial twofold decrease of Mg^{++} to 0.125 M, there was a thousandfold destruction of virus.

Immediately after mixing, aliquots of the Mg-virus mixtures used in the experiment described above were placed in a 37° C. incubator, and samples were tested at longer intervals. Fig. 3

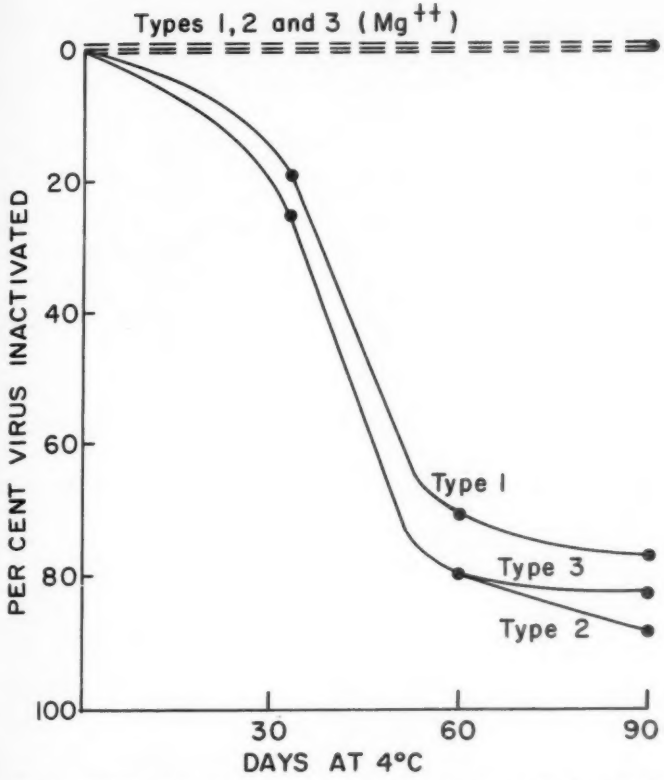


FIG. 4. Stabilization of attenuated polioviruses by molar Mg^{++} at 4° C.

illustrates the stabilizing effect of Mg^{++} at various concentrations after prolonged storage of the virus at 37° C. After 24 hours, the virus stored at 37° C. in high concentrations of $MgCl_2$ showed no loss in titer, as compared to the control stored in H_2O, in which 50% of the virus population was inactivated. After three days at 37° C., the virus showed no loss of infectivity in 1 M Mg^{++}, but there was increasing inactivation as virus was stored in Mg^{++} concentrations below this molarity. The control (without additional Mg^{++}) showed 99% of the virus inactivated.

Sparing Effect of Mg^{++} on Dilute Suspensions of Virus.— Type 1 attenuated virus was diluted in M-E medium to contain

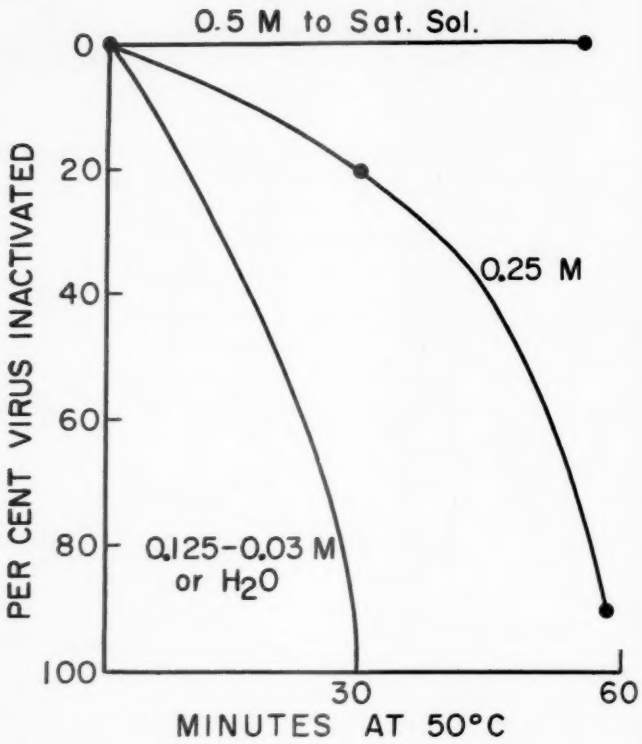


FIG. 5. Effect of Mg^{++} concentration on stability of type 1 poliovirus at 50° C.

10^6, 10^4, 10^3, and 10^2 PFU/ml in 1 M $MgCl_2$. These tubes and a duplicate series without added Mg^{++} were heated for 1 hour at 50° C. All Mg-virus samples survived the heating without any loss of infectivity, while the series without added Mg^{++} showed complete inactivation. The dose of virus selected for use in oral poliovirus vaccine is from $10^{5.0}$ to $10^{5.8}$ PFU/ml. Thus, attenuated virus at the dilutions used, or suggested for use, in the vaccine is completely protected against thermal inactivation at 50° C. for 1 hour.

With the use of decreasing concentrations of Mg^{++} (from 2 M to 0.06 M in twofold dilutions), the inactivation curves for

virus at 10^7 and 10^4 PFU/ml at 50° C. for 1 hour were parallel. The inactivation curves for the high 10^7 concentration of virus, already presented in Fig. 5, represent the results for the low 10^4 concentration as well. Thus there are no natural products (proteins or other substances) from the infected cell cultures which play a role in protection afforded by Mg ions. If they existed, such natural stabilizers would be present at higher concentration in the more concentrated virus suspension, which would then be more readily stabilized by the added $MgCl_2$. However, no differences were found.

Effect of Mg^{++} at Temperatures above 50° C.—Although 1 M Mg^{++} stabilized polioviruses at 50° C., the same did not hold true for higher temperatures. Virus (10^7 PFU/ml in M Mg^{++}) was placed in a 50° C. water bath for 5 minutes, and then immediately transferred to a 55° C. (or 60° C.) bath for an additional 5 minutes. Thermal inactivation was stopped by plunging the tubes into an ice-water bath. Heating for 5 minutes at 55° C. resulted in a loss of 90 per cent of virus, and at 60° C., 99.9 per cent. Control virus was completely inactivated after 5 minutes at 55° C. (see Table 2).

TABLE 2

Effect of Mg ions on thermal stability of type 1 attenuated poliovirus (10^7 PFU/ml)

Temperature	Log_{10} virus inactivated (PFU/ml) after 5 minutes	
	1 M Mg^{++}	H_2O
50° C.	0	4.3
55° C.	1	>5.0
60° C.	3	..
65° C.	>5.0	..

Prolonged Heating of Type 1 Attenuated Virus at 50° C.—Virus (10^7 PFU/ml) in M $MgCl_2$ was heated at 50° C., and control virus without added $MgCl_2$ was heated at the same temperature. Samples were obtained at 24 hours, and for 5 additional days thereafter, and assayed by plaque counting. At 24 hours (see Fig. 6), 1 log of virus was inactivated in the M $MgCl_2$ series; at 2 days, 2.6 logs; at 3 days, 3.8 logs; at 4 days, 4.2 logs; at 5 days, 4.4 logs; and at 6 days no infectivity could be detected.

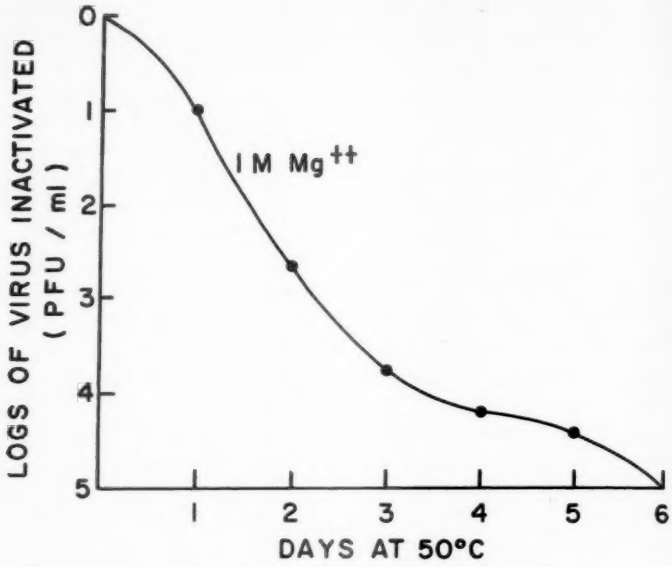


FIG. 6. Inactivation of type 1 virus at 50° C. over a 6 day period.

With the attenuated vaccine strains, no detectable virus was present in the control without added $MgCl_2$ after heating for one hour.

Comparison of Standard Diluents and Mg^{++} as Stabilizing Agents for Attenuated Poliovirus at 50° C.—Since high concentrations of Mg^{++} exhibited a remarkable sparing effect on type 1 poliovirus at 50° C., the protective effect of this ion was compared with that exerted by some commonly used stabilizing agents. As shown in Fig. 7, at 50° C. for 1 hour, nutrient broth, skim milk, and bovine albumin protected only about one of every 1000 PFU from inactivation, whereas 1 M Mg^{++} protected the entire viral population.

Comparison of L-Cystine and Mg^{++} as Stabilizing Agents for Type 1 Attenuated Poliovirus at 50° C.—Pohjanpelto(20) has reported that if a heat-sensitive strain of poliovirus is pretreated with 50μg/ml L-cystine at 37° C. for 6 hours, and then heated at 50° C. for 1 hour, 80% of the virus may be inactivated, while in the absence of cystine over 99.9% of the virus is killed. We

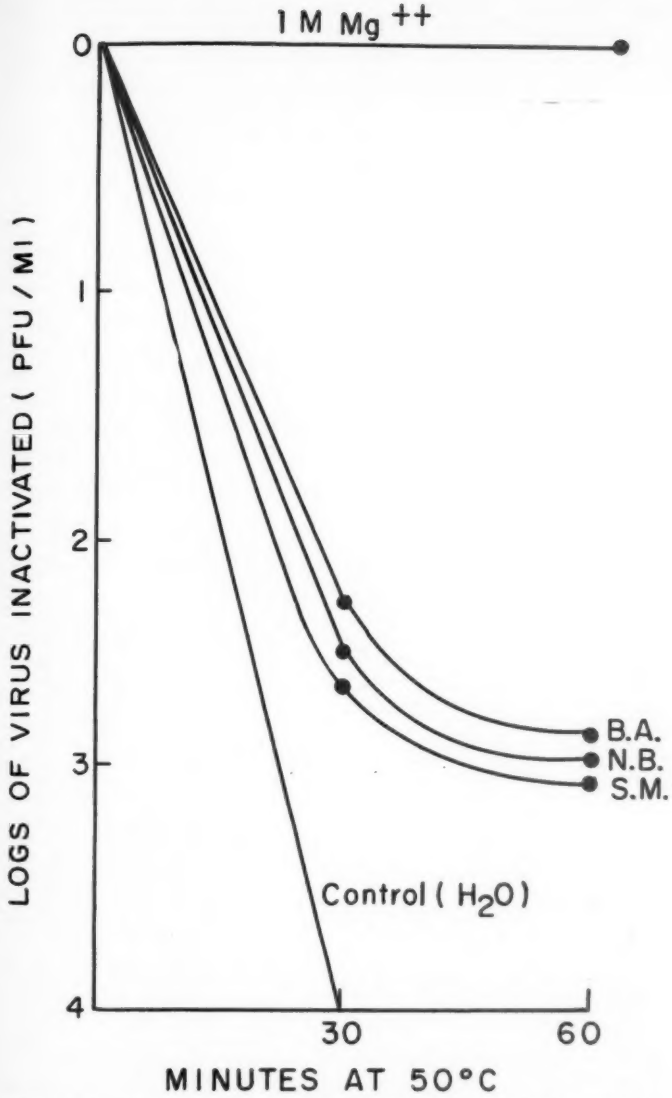


FIG. 7. Comparison of standard diluents and Mg^{++} as stabilizing agents for attenuated poliovirus at 50° C. Type 1 virus (10^7 PFU/ml) in M Mg^{++}, B.A. = 10% crystalline bovine albumin (Armour); N.B. = nutrient broth (Difco); S.M. = 50% skim milk (Pet); control in distilled water.

compared L-cystine and $MgCl_2$ as thermal stabilizing agents. The type 1 LSc attenuated poliovirus used in these experiments is a heat-sensitive strain.

Aliquots of virus (at 10^7 PFU/ml) in 1 M Mg^{++}, 50 μg L-cystine/ml, and a control without any additives were placed at 37° C. for 6 hours. A second control of untreated virus was immediately frozen for reference. At the end of 6 hours at 37° C., the tubes were placed at 50° C., and aliquots were obtained at intervals of 15, 30, 60, and 120 minutes. The results, illustrated in Fig. 8 show that about 95% of the virus in the cystine aliquot was inactivated after 60 minutes at 50° C., and over 99.9% at 120 minutes, as contrasted to no loss of virus infectivity in the Mg^{++} aliquot even after 2 hours' exposure to 50° C. Similar observations were made using virulent type 1 (Mahoney) virus.

The Effect of Different Concentrations of Na^+ and K^+.—At final concentration of 2 M Na^+ or 1 M K^+, no inactivation of

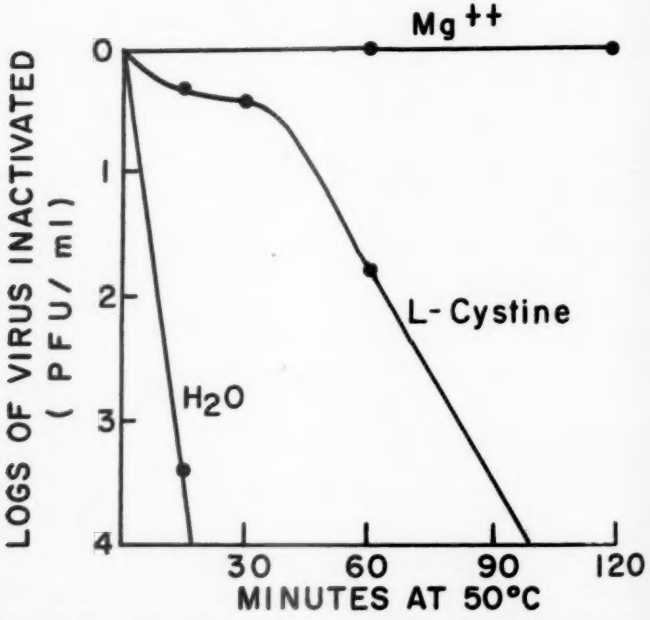


FIG. 8. Comparison of M Mg^{++} and L-cystine as stabilizing agents for type 1 attenuated virus.

virus (type 1 attenuated) was detectable after 30 minutes at 50° C., but with twofold decreasing concentrations of these monovalent salts, inactivation became progressively more pronounced, as shown in Fig. 9, left.

However, aliquots of the same monovalent salt-virus mixtures placed at 37° C. for a period of 6 days showed opposite effects (see Fig. 9, right). At 37° C. the virus survived to a much greater extent in dilute saline or even in water than it did in 2 M NaCl. After 6 days in 2 M NaCl, less than 0.001% of the virus remained, as compared to 1% in H_2O or dilute salt concentrations (0.06 M). Tests with KCl at 1 M or less yielded the same results as for NaCl.

Thus, although Mg^{++} and Na^{++} in high concentrations spared type 1 poliovirus at 50° C. for 30 minutes, the cations had opposite effects at 37° C. At the lower temperature, M Mg^{++} was still protective, but 2 M Na^{+} was actually destructive. That inactivation of virus at 50° C. proceeds differently from that at 37° C. has already been suggested by Youngner (29).

"Dissociation" of the Stabilized Magnesium-Virus Mixture by Dilution.—Type 1 attenuated poliovirus at 10^7 PFU/Ml in M $MgCl_2$ was incubated for 30 minutes at 25° C. A control virus suspension without $MgCl_2$ was incubated under the same conditions. Both samples were then diluted 100-fold, and heated at

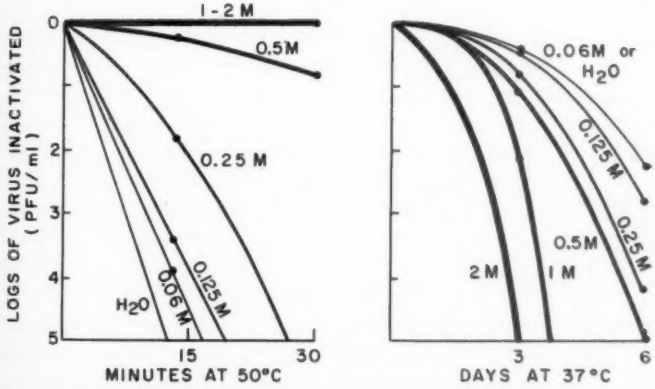


FIG. 9. Stabilization of poliovirus (type 1 attentuated) by high concentrations of NaCl at 50° C. but inactivation at 37° C. The thicker the line, the higher the molarity of NaCl.

50° C. for 5 minutes. The rates of inactivation were similar in both cases, yielding the values expected for heating in water alone, with over 99% of the virus being inactivated. Thus, no thermal sparing effect was exhibited by virus which had received prior incubation with M $MgCl_2$ at low temperatures.

Experiments with Mg^{++} Protected Survivors.—Heating of type 1 attenuated polioviruses (10^7 PFU/ml) at 50° C. for 16 hours in Mg^{++} inactivated only 1.5 $\log_{10}$ of virus. Survivors of such prolonged heating were passed in MK cells, and at the same time control virus, which had not been exposed to heat, was passed. The resultant virus harvest at $10^{6.5}$ PFU/ml was tested for resistance to heating at 50° C. in the presence and in the absence of M $MgCl_2$. The rates of inactivation were similar with both viruses. Thus no unusual, heat-resistant progeny were produced by heating in the presence of $MgCl_2$.

Identification of Heat-Resistant Survivors as Intact Virus Particles and Not Infectious RNA.—Survivors of heating for 16 hours at 50° C. in 1 M Mg^{++} were mixed with equal volumes of (a) RNAase, (b) type 1 antiserum (inactivated at 60° C. for 2 hours), and (c) water. These aliquots were tested for virus. No plaques were evident in the sample containing type-specific serum. The other two samples had the same PFU counts. Therefore, only whole virus, capable of reaction with specific antiserum, was present among the survivors, and no viral nucleic acid free of its protein capsid was detectable.

Genetic Stability of Virus Stored or Heated with M $MgCl_2$.—Attenuated viruses (types 1, 2, and 3) were stored for 3 weeks at room temperature in M $MgCl_2$, or heated in M $MgCl_2$ at 50° C. for 90 minutes, and no loss in viral titer was observed. The viruses were then tested for d and T markers, and for intracerebral neurovirulence in monkeys. In all of these marker tests, the Mg-treated viruses after storage or heating behaved as do the original vaccine strains (4, 22, 17, 16).

Discussion

Our findings reported herein show that cations in general protect polioviruses from thermal inactivation. The most advantageous ion studied was Mg^{++}, which was used as $MgCl_2$ or $MgSO_4$. In addition to its high stabilizing properties, Mg^{++} at one molar concentration showed the least cytotoxicity of any of the cations

studied. It had the further advantage of not producing sediments in poliovirus suspensions. The degree of protection of oral poliovirus vaccine by M $MgCl_2$ is remarkable; the strains in the vaccine are completely protected for at least 2 hours at 50° C., for at least 3 days at 37° C., for at least 25 days at room temperature, and for at least 3 months at 4° C.

Our results with other viruses show that the Mg ions also protect other RNA-containing viruses from thermal inactivation, but enhances the heat inactivation of DNA-containing viruses (26).

Pollard (21) has suggested that heating a virus may bring about basic structural alterations of the particle due to the differential expansion of the various parts of the virus under the action of heat. This change, he states, might well disrupt the spatial relationships that are necessary to retain the structural integrity of the virus. It may well be that cations in high molar concentration permeate the virus particle and reinforce the structure during heat stress.

The use of M $MgCl_2$ as a stabilizing agent for poliovirus has manifold advantages. Of immediate value is the fact that Mg^{++} in molar concentration can be used to protect live poliovirus vaccines in their commercial use in the field or in storage in the ordinary refrigerator for prolonged periods of time. Mg ions (1 M) can be incorporated into the final dosage form, or the concentrated vaccine can be preserved with M Mg^{++} and diluted as needed. There are no contraindications to the use of Mg^{++} in the amounts required (1 ml of M Mg^{++} or less) for stabilizing an individual dose. $MgCl_2$ at molar concentration is inhibitory (and considerably bacteriocidal) to most forms of bacterial and fungal life (27), and additional preservatives may not be necessary during storage of the virus, or at transit temperatures.

Another area where high cation concentrations might be of value is in the preservation of poliovirus during its inactivation by formalin. The present production process for killed poliomyelitis vaccine is to incubate virus plus dilute formalin at 37° C. for many days (23). Therefore a considerable amount of virus inactivation takes place by heat (see Fig. 3 for heat inactivation of poliovirus at 37° C.). Thus only a fraction of the virus is inactivated by the formalin. In the presence of high concentrations of salts as shown in the present study, the virus should be in-

activated by formalin alone, and the resultant vaccine should be of higher potency. The salt will have to be removed but it is a simple matter to remove the excess salt by dialysis; or the inactivated virus vaccine can be removed from the reaction mixture containing the salts.

Summary

The use of high concentrations (one molar) of divalent cations is described as a means of stabilizing poliovirus. Mg^{++} in molar concentration protects poliovirus at 50° C. for 1–2 hours, with only gradual loss of infectivity over the next 24 hours. Molar Mg^{++} protects poliovirus at room temperature (25° C.) for 25 days, and at 37° C. for over 3 days, without any detectable loss of infectivity. M Mg spares poliovirus for at least 90 days at 4° C. without any loss of infectivity.

NaCl at 1 or 2 molar concentration protects poliovirus at 50° C. for shorter periods (30–60 minutes), but at 37° C. it enhances viral inactivation.

The advantages of molar Mg^{++} as a stabilizing agent for poliovirus are many. $MgCl_2$ can protect live poliovirus from thermal inactivation in its field use and in storage. $MgCl_2$ can preserve the vaccine in its final dosage form or in concentrates to be diluted as needed. Molar $MgCl_2$ is inhibitory to bacterial and fungal life, and thus the addition of preservatives may not be necessary during storage or shipment of poliovirus. The use of bulky and expensive refrigerants is no longer needed in shipping poliovirus vaccines (even overseas), and no special attention need be paid to temperatures prevailing while vaccine is in transit in ordinary mail. Molar Mg^{++} should also be useful in preparation of killed poliomyelitis vaccines of higher potency.

REFERENCES

1. Adams, M. H.: Stability of bacterial viruses in solutions of salts. *J. Gen. Physiol.*, 32: 579, 1949.
2. Allen, E. G., B. Kaneda, A. J. Girardi, T. F. M. Scott, and M. M. Sigel: Preservation of viruses of the psittacosis-lymphogranuloma venerum group and herpes simplex under various conditions of storage. *J. Bact.*, 63: 369, 1952.
3. Allisbaugh, H. C.: Patent No. 2,787,577, Stable smallpox vaccine, U.S. Patent Office, April, 1957.
4. Benyesh-Melnick, M., and J. L. Melnick: The use of in vitro markers and monkey neurovirulence tests to follow the genetic changes in attenuated poliovirus multiplying in the human alimentary tract. *Pan. Am. San. Bur. Sci. Publ.*, 44: 179, 1959.

5. Burnet, F. M., and M. McKie: Balanced salt action as manifested in bacteriophage phenomena. *Austral. J. Exp. Biol. Med. Sci.* 7: 183, 1930.

6. Grossowicz, N., A. Mercado, and N. Goldblum: A simple method for concentration of live and formaldehyde-inactivated poliovirus. *Proc. Soc. Exp. Biol. Med.*, 103: 872, 1960.

7. Holland, J. J., B. H. Hoyer, L. C. McLaren, and J. T. Syverton: Enteroviral ribonucleic acid. I. Recovery from virus and assimilation by cells. *J. Exp. Med.*, 112: 821, 1960.

8. Hsiung, G. D., and J. L. Melnick: Plaque formation with poliomyelitis, Coxsackie and orphan (ECHO) viruses in bottle cultures of monkey epithelial cells. *Virology*, 1: 533, 1955.

9. Kaplan, A. S., and J. L. Melnick: Effect of milk and cream on the thermal inactivation of human poliomyelitis virus. *Amer. J. Publ. Health*, 42: 525, 1952.

10. Kaplan, A. S., and J. L. Melnick: Effect of milk and other diary products on the thermal inactivation of Coxsackie viruses. *Amer. J. Publ. Health*, 44: 1174, 1954.

11. Koch, G.: Influence of assay conditions on infectivity of heated poliovirus. *Virology*, 12: 601, 1960.

12. Lark, K. G., and M. H. Adams: The stability of phages as a function of the ionic environment. *Symposia on Quantitative Biology*, 18, 171, 1953.

13. Medearis, D. N., J. H. Arnold, and J. F. Enders: Survival of polioviruses at elevated temperatures (60–75° C.). *Proc. Soc. Exper. Biol. Med.*, 104: 419, 1960.

14. Meiklejohn, G., E. Rust, and O. Brunetti: Comparison of diagnostic methods for Western Equine and St. Louis encephalitis. *J. Immunol.*, 62: 293, 1949.

15. Melnick, J. L.: Tissue culture techniques and their application to original isolation, growth, and assay of poliomyelitis and orphan viruses. *Ann. N.Y. Acad. Sci.*, 61: 754, 1955.

16. Melnick, J. L., and J. C. Brennan: Monkey neurovirulence of attenuated poliovirus vaccines being used in field trials. *Pan. Am. San. Bur. Sci. Publ.*, 44: 65, 1959.

17. Murray, R., R. Kirschstein, G. Van Hoosier, Jr., and S. Baron: Comparative virulence for rhesus monkeys of poliovirus strains used for oral administration. *Pan. Am. San. Bur. Sci. Publ.*, 44: 39, 1959.

18. Norman, A., and R. C. Veomett: Heat inactivation of poliovirus ribonucleic acid. *Virology*, 12: 136, 1960.

19. Olitsky, P. K., R. H. Yager, and L. C. Murphy: Preservation of neurotropic viruses. *U.S. Armed Forces Med. Jour.*, 1: 415, 1950.

20. Pohjanpelto, P.: Stabilization of poliovirus by cystine. *Virology*, 6: 472, 1958.

21. Pollard, E. C.: Theory of the physical means of the inactivation of viruses. *Ann. N.Y. Acad. Sci.*, 83: 654, 1960.

22. Sabin, A. B.: Recent studies and field tests with a live poliovirus vaccine. *Pan. Am. San. Sci. Publ.*, 44: 14, 1959.

23. Salk, J.: Poliomyelitis vaccine in the fall of 1955. *Am. J. Publ. Health*, 46: 1, 1956.

24. Wallis, C., R. T. Lewis, and J. L. Melnick: Preparation of kidney cell cultures. *Tex. Rep. Biol. Med.*, 19: 194, 1961.

25. Wallis, C., and J. L. Melnick: Magnesium-chloride enhancement of cell susceptibility to poliovirus. *Virology*, in press.

26. Wallis, C., and J. L. Melnick: Cationic inactivation of DNA vacuolating virus in RNA poliovirus suspensions. *Tex. Rep. Biol. Med.*, 19: 701, 1961.

27. Wilson, G. S., and A. A. Miles: Topley and Wilson Principles of Bacteriology and Immunology (Williams and Wilkins), 4th edition, 135, 1955.

28. Woese, C.: Thermal inactivation of animal viruses. *Ann. N.Y. Acad. Sci.*, 83: 741, 1960.

29. Youngner, J. S.: Thermal inactivation studies with different strains of poliovirus. *J. Immunol.*, 78: 282, 1957.

CATIONIC INACTIVATION OF VACUOLATING VIRUS (SV_{40}) IN POLIOVIRUS SUSPENSIONS*

CRAIG WALLIS AND JOSEPH L. MELNICK

Introduction

Virus for vaccines is usually grown in primary cell cultures (for example, poliovirus in monkey kidney cultures). In the process, viruses present as spontaneous contaminants of the cell cultures are inadvertently incorporated into the virus harvests from which the vaccine is prepared. In the case of oral, live poliovirus vaccine, the contaminating virus may be carried along with the vaccine virus and find its way into the final vaccine. Thus many lots of live poliovirus vaccine, particularly those prepared from rhesus monkey (*Macaca mulatta*) kidney cultures have contained relatively high concentrations of vacuolating virus (4) (as high as 10^5 TCD_{50} per ml of vaccine). Vacuolating virus is more resistant to treatment with formalin than the poliovirus (1) and has been found as a live contaminant of Salk-type vaccines also.

In an accompanying report (6) we described the stabilization of poliovirus with divalent cations. The present paper is concerned with the inactivation of extraneous vacuolating virus (SV_{40}) from poliovirus vaccine by the use of high ionic concentrations of divalent cations. Such salt solutions which stabilize poliovirus at 50° C. for 1 hour were found to inactivate the vacuolating virus within a few minutes at the same temperature.

Materials and Methods

Monkey kidney (MK) cells. Kidneys from immature rhesus monkeys were trypsinized, grown, and maintained in Melnick's medium as described in detail elsewhere (2, 5). Kidney cultures from young African green (grivet) monkeys, *Cercopithecus aethiops sabaeus*, were also prepared in the same manner.

Viruses. The strain of vacuolating virus (SV_{40}), was isolated from Sabin's original large lot of oral poliovaccine (3), and was maintained by passage in African green monkey kidney cultures. Type 1 attenuated poliovirus was the

* Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas.

LSc strain used in the oral poliovaccine (Sabin), passed once in MK cultures in this laboratory.

Virus assays. All virus titrations were carried out by determination of plaque-forming units (PFU), using the bottle technique.

Medium for overlay for SV_{40} consisted of Earle's salt solution, 0.1% bovine albumin, 0.1% yeastolate (Difco), 0.4% $NaHCO_3$, in a final concentration of 1.5% agar. A second overlay at 10 days was made consisting of the same ingredients and incorporating 1:30,000 neutral red. Overlay for poliovirus has been described elsewhere (6).

Heating methods. Tissue culture fluids containing virus were mixed with an equal volume of the salt or diluent under test. Before it was mixed with virus, the salt was at twice the final concentration desired.

Aliquots to be heated were delivered directly to the bottom of tubes, and the top of each tube was flamed thoroughly to sterilize at least one inch of the uppermost surface of the tube. All test tubes used were uniform in size and thickness, measuring 13 × 100 mm, with an 0.8 mm wall. The tubes were then rubber-stoppered and plunged into the water bath, covering the tube to ¼-inch of the stopper. At the end of the heating period, the tubes were immediately transferred to an ice-water bath.

Viruses in M Mg^{++} or M Ca^{++} could be diluted tenfold and plated on monolayers without toxic effect upon cells. Before plating undiluted virus-salt mixtures, these samples were dialyzed for 2 hours against cold running water to reduce the salt concentration.

Results

Effect of M Mg^{++} or M Ca^{++} on vacuolating agent (SV_{40}) at 50° C. SV_{40} was diluted in (a) M Mg^{++}, (b) M Ca^{++} and (c) distilled water, to contain 10^6 PFU/ml. Samples were heated at 50° C. for 5, 15, 30 and 60 minutes. As illustrated in Fig. 1, vacuolating agent SV_{40} diluted in distilled water was remarkably stable at 50° C. for 60 minutes, with loss of only a small percentage (5%) of virus. However, the agent diluted in M Mg^{++} or M Ca^{++} lost 90% of its infectivity after 5 minutes at 50° C. in either of these cations. After 15 minutes at this temperature, SV_{40} was not detectable in the Mg^{++} or Ca^{++} samples. When stored in M Mg^{++} or M Ca^{++} at room temperature (25° C.), there was a slight loss in infectivity after one hour. However, at 2 M concentration, inactivation of vacuolating virus proceeds relatively rapidly even at 25° C.

Effect of cations on mixtures of poliovirus and vacuolating agent SV_{40}. Type 1 attenuated poliovirus was mixed with equal volume of SV_{40} so as to contain 10^7 and 10^6 PFU/ml, respectively. This virus mixture was diluted with equal volume of (a) 2

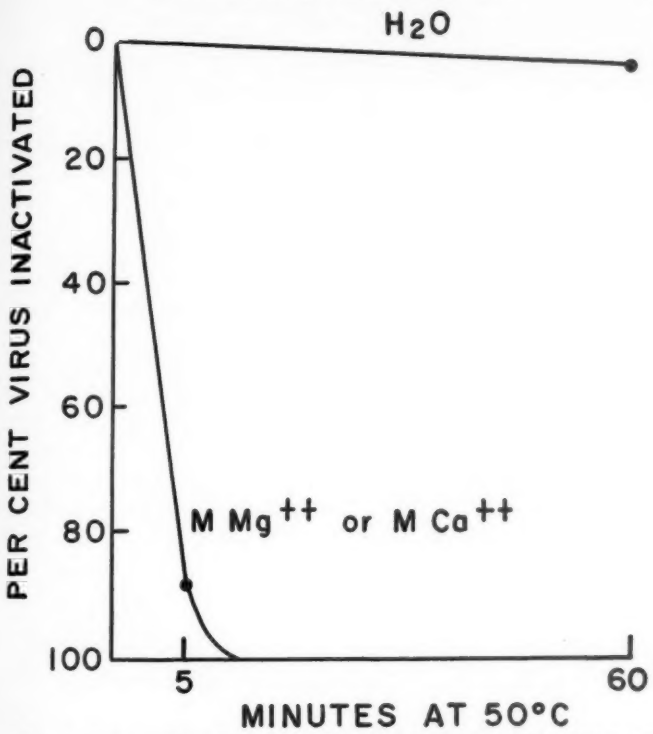


FIG. 1. Enhanced inactivation of SV_{40} by M Ca^{++} or M Mg^{++} at 50° C. SV_{40} was diluted to contain 10^6 PFU/ml in molar concentration of divalent cation. M Mg^{++} and (b) distilled water. Samples were heated at 50° C. for 5, 30, and 60 minutes.

Fig. 2 shows that the poliovirus was stabilized by $MgCl_2$ for 60 minutes at 50° C. without any detectable loss, in contrast to its lability in water. This is represented by the "M Mg^{++}" and "H_2O" curves, respectively. Titrations on rhesus cells resulted in plaques that formed between the 2nd and 3rd day, and were counted as polio plaques, since SV_{40} did not produce plaques on rhesus cells.

A sample of the virus mixture diluted in water was treated with a pool of Type 1 polio sera free of vacuolating agent antibody. The sample was then titrated in grivet cells and SV_{40} plaques formed between the 10th and 14th days.

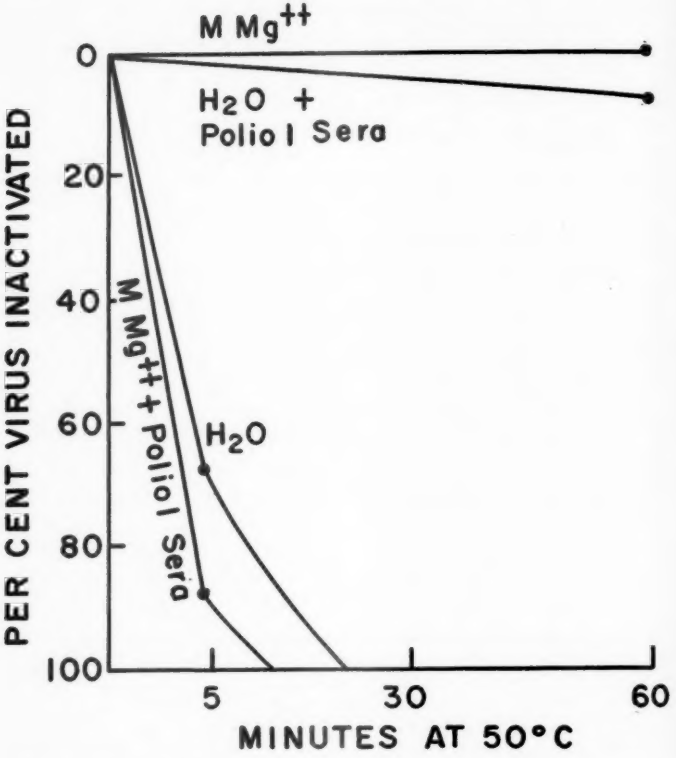


FIG. 2. SV_{40} and poliovirus (attenuated Type 1) mixed to contain 10^6 and 10^7 PFU/ml, respectively. Mixed virus suspension diluted with equal volume of (a) 2 M Mg^{++}, (b) distilled H_2O. Detection of surviving poliovirus was made by plating on rhesus monkey kidney cells, and counting PFU developing in 2 to 3 days. SV_{40} was detected by neutralizing poliovirus with a pool of Type 1 polio antisera (free of vacuolating virus antibody), and plating on African green monkey kidney cells. SV_{40} plaques were read between the 11th–14th days.

In Fig. 2, the stability of SV_{40} diluted in water at 50° C. for 1 hour is represented by the curve labeled "H_2O + polio 1 sera." The enhanced inactivation of vacuolating agent SV_{40} is represented by the "M Mg^{++} + polio 1 sera" curve. There was a loss of 90% infectivity after 5 minutes at 50 °C., with no detectable infectivity at 30 minutes.

Discussion

Currently, the problem of producing either live poliovirus vaccines or formalinized vaccines is complicated by the presence of extraneous viruses of the vacuolating virus type (SV_{40}). The stabilizing effect of M Mg^{++} or M Ca^{++} in preventing the inactivation at 50° C. of poliovirus (a ribonucleic acid-containing virus), and the opposite effect of these divalent cations on vacuolating virus (a deoxyribonucleic acid-containing virus), affords a solution to this problem. Thus preparations which contain the desired poliovirus in the course of manufacturing vaccine, but which also contain the contaminant vacuolating virus, can be freed of infective vacuolating virus. By adding 1 or 2 M $MgCl_2$, heating at 50° C. for 1 hour, and then cooling to 4° C., the vacuolating virus can be inactivated without affecting the poliovirus. The resulting polio vaccine product containing the molar cation is stable at 4° C. for at least several months (6), and needs no further preservative because of the bacteriocidal and bacteriostatic effects of the cation at this molarity (7).

Summary

The use of 1 to 2 molar $MgCl_2$ is described as a means of stabilizing poliovirus suspensions at 50° C. while inactivating viruses of the vacuolating virus type which may be present as contaminants in the same suspension.

REFERENCES

1. Goffe, A. P., J. Hale, and P. S. Gardner: Poliomyelitis vaccines. *Lancet*, 1: 612, 1961.
2. Melnick, J. L.: Tissue culture techniques and their application to original isolation, growth and assay of poliomyelitis and orphan viruses. *Ann. N.Y. Acad. Sci.*, 61: 754–772, 1955.
3. Sabin, A. B.: Properties and behavior of orally administered attenuated poliovirus vaccine. *J. Am. Med. Assoc.*, 164: 1216–1223, 1957.
4. Sweet, B. H., and M. R. Hilleman: The vacuolating virus, SV_{40}. *Proc. Soc. Exptl. Biol. and Med.*, 105: 420–427, 1960.
5. Wallis, C., R. T. Lewis, and J. L. Melnick: Preparation of kidney cell cultures. *Tex. Reports Biol. Med.*, 19: 194–197, 1961.
6. Wallis, C., and J. L. Melnick: Stabilization of poliovirus by cations. *Tex. Reports Biol. and Med.*, 19: 683–700, 1961.
7. Wilson, G. S., and A. A. Miles: Topley and Wilson Principles of Bacteriology and Immunology (Williams and Wilkins), 4th edition, 135, 1955.

NOTE

NOTE

A NEW MOUSE STRAIN SUSCEPTIBLE TO MAMMARY CANCER

ALFRED TAYLOR AND GEORGE F. MCKENNA[a]

Animals susceptible to cancer are a necessity for most investigations of the cancer problem. It is especially important to have available animals characterized not only by a high incidence of spontaneous tumors but which are also resistant to noncancerous diseases. The C3H mouse strain developed originally by Dr. L. C. Strong at Yale University certainly qualifies on the basis of susceptibility to spontaneous mammary cancer, but it does tend to have a low resistance to *Salmonella* infection. Nevertheless this strain has proved to be most valuable in cancer research and is used in numerous laboratories.

A C3H line of mice, originally obtained from Dr. Andervont, of the National Cancer Institute, has been maintained in our laboratory by brother × sister mating for more than fifteen years. About ten years ago a mutant occurred in our C3H colony which differs in a number of characteristics from the source stock. These mice qualify as an inbred strain in accordance with the standards set up by The Committee on Standardized Genetic Nomenclature for Mice (1) since they have been maintained by brother × sister matings for more than twenty generations. This new mouse strain has been designated C3HBL, since it is a black variant of the original C3H line. However, change in color has been accompanied by differences in some other characteristics. The new mouse, in relation to the source strain, is characterized by increased disease resistance, a slightly heavier body and greater activity. Susceptibility to the spontaneous development of mammary adenocarcinoma is equal to that of our C3H mice. There is also present in this new strain an increased tendency towards the development of multiple mammary tumors, and the growth of the spontaneous tumors is more rapid than is true for the tumors occurring in our C3H mice.

[a] From the Clayton Foundation Biochemical Institute, The University of Texas, Austin. Submitted for publication March 10, 1961.

The new strain is slightly heavier at all ages as compared with the C3H mice, and when kept in cages which do not permit exercise, obesity is frequent. Weight averages for the two strains at 1, 5 and 10 months of age, in grams, are for the C3H: 17.5, 26.2 and 32.0; and for the C3HBL: 18.5, 30.6 and 35.1. The average age at which spontaneous mammary tumors develop is about the same for the two strains. Tumors appeared in virgin C3H and C3HBL at 51 and 53 weeks, and for breeders the figures were 33 and 34 weeks respectively. The total incidence of mammary adencarcinoma ranges from 88 to 95% for both of these mouse strains in our colonies.

The two strains have been tested for their reaction to the use of a voluntary exercise wheel. The C3HBL strain, using both males and females, exercised about 20% more per day in tests of 5 days duration as compared with C3H males and females. Also, the C3HBL mice are comparatively difficult to restrain for injection.

For cancer research requiring a mouse with high incidence of spontaneous mammary tumors, good resistance to disease, and which breeds readily, the C3HBL strain may in some instances be preferable to the source C3H strain.

REFERENCE

1. Committee on Standardized Genetic Nomenclature for Mice. Standardized nomenclature for inbred strains of mice: Second listing. *Cancer Research*, 20: 145–169, 1960.

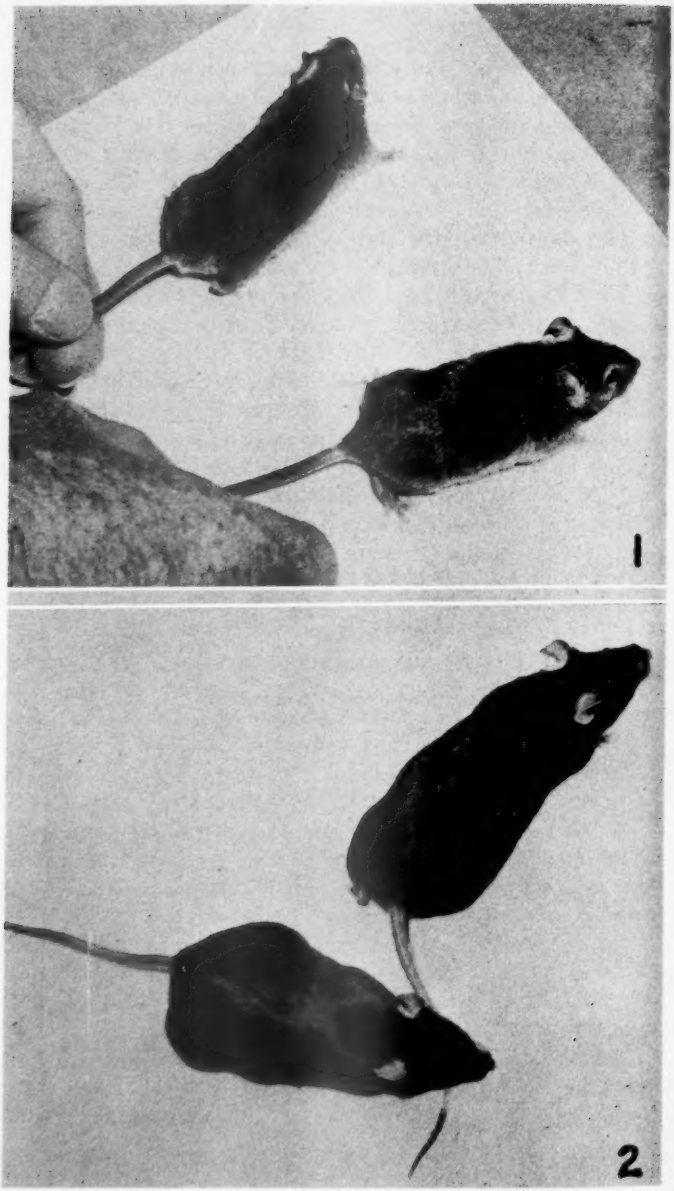


FIG. 1. C3H mice from our colony.
FIG. 2. C3HBL mice. Upper, ♂. Lower, ♀.

CALLING ATTENTION TO

CALLING ATTENTION TO

FEBRUARY, 1961

Anesthesia: H Abramson edits important volume on *Resuscitation of the Newborn Infant* (CV Mosby, St Louis 3, Mo, 1960, 274pp, $10). VJ Collins gives *Fundamentals of Nerve Blocking* (Lea & Febiger, Philadelphia 6, 1960, 354pp, 144 illus $9.5). FT Evans & C Gray well edit *General Anesthesia*, Vol 1 on basic principles; Vol 2 on techniques, special fields & hazards (Butterworth, Washington 14, DC, Vol 1, 1960, 545pp, Vol 2, 443pp, 192 illus, $19.5). LM Monheim describes *General Anesthesia in Dental Practice* (CV Mosby, St Louis 3, Mo, 1960, 461pp, 114 illus, $10). RM Smith writes *Anesthesia for Infants & Children* (Mosby, St Louis, 1960, 418pp, 182 illus, $12).

Carbon-Dioxide: Note significant symposium edited by JC Eckenhoff, covering reviews of biological role, production, storage, transport & removal from mammalian bodies, relation to body systems & to anesthesia (*Anesthesiol* 21/585–766/60). A Pirson revises Vol 5, *Handbuch de Pflanzephysiologie*, on *The Assimilation of Carbon-Dioxide*, with 70 contributions (Springer, W Berlin, 1960, 1882pp, 640 illus, DM 530).

Cardiovascular: F Allodi makes comprehensive survey of cardiac biology in *Cardiobiologia* (LS Olschki, Firenze, Italy, 1960, 988pp, 154 illus, 10,000 lire). I Welt compiles monumental *Index-Handbook of Cardiovascular Agents*, giving references to actions of all cardiovascular chemicals arranged both by actions & by agents; the 1st issue covering 13,427 reports from 1950 to 1955, National Academy Sciences, Washington, DC, 1960, 1623pp in 2 pts, $15; Vol 1 for 1931–50, & Vol 3 for 1955–1960 will appear during next 2 years, & then annually.

General: WO Fenn offers front seats for biologists (*AIBS Bull*, 10/13/60); PC Clark sketches background & gives pleasant accounts of *Pioneer Microbiologists of America* (Univ Wisconsin Press, Madison, 1961, 383pp, 38 portraits, $6). R Marx well considers *The Health of the Presidents* (GP Putnam's Sons, New York, 1961, 238pp, $5). W Rubin edits interesting symposium on culture, society & health, in which R Fernandez-Marin & VM Eckhardt of Puerto Rico describe schizophrenogenesis by symbolic logic (Ann NY Acad Sci, 84/783–1060/Dec 8/60).

Preclinical: BB Brown analyses central nervous system drug action in mice (*Arch Internat Pharmacody Therap*, 128/391/60). AS Gordon & Co discuss humoral regulation of leucocyte number (*Trans NY Acad Sci*, series II, 231/39/60). DE Green & Y Hatefi discuss energy transduction by mitochondria in cells (*Science* 133/13/Jan 6/60). SE Jacobs & Co sympose on chemical disinfection. (*J. Pharm Pharmacol*, Suppl Vol 12, 9T–40T, Dec/60). R Katzman asks if glia are high sodium cells in discussing electrolyte distribution in mammalian central nervous system (*Neurology* 11/27/61). LP Lipchina & Co find inhibitors of free radical reactions (like propylgallate) inhibit mitosis in human cancer cells (*Doklady Biol Sci Sect*, 131/204/60). B Metz discusses brain acetylcholine enzyme system in respiratory control

(*Neurology* 11/37/61). LA Rapoport & RG Kostyenovskii discuss mutagenic activity of organic phosphate inhibitors of cholinesterase (*Doklady Biol Sci Sect*, 131/246/60). EL Way & TK Adler describe metabolism of morphine & its relative (*Pharmacol Rev*, 12/383/60). EK Zhukov & EB Kossovaskaia honor 75th birthday of AN Krestovnikov by arranging symposium on 17 articles on physiology of exercise & work (*Sechenov Physiol J USSR* 46/907–1041/60). RD Zilversmit & Co note plasmalogen in human blood platelets (*J Biol Chem*, 236/47/61).

Clinical: H Friebel & S Sommer note aryl-sultam compounds are anti-convulsive & sedative, one ("Ospblot"!) useful in epilepsy (*Deutsch Med Wchnschr*, 85/2192/Dec 9/60). F Hahn surveys analeptic drugs (*Pharmacol Rev*, 12/447/60). S Margolin finds carisoprodol 5 times as active as aspirin in raising tooth-pain threshold (*Proc Soc Exp Biol Med*, 105/531/60). CA Nau, J Neal & VA Stembridge report on physiological effects of carbon black (*Arch Environ Med*, 1/512/60). RE Olson & Co find evidence for defect in tryptophan metabolism in chronic alcoholism, confirming RJ Williams' ideas (*New Eng J Med*, 263/1069/Dec 8/60). G Rooth & Co discuss CO_2 tension & acid-base balance of amniotic fluid (*Amer J Obs Gyn*, 81/1,4/61). H Selye & Co note excessive stimulation of salivary gland growth by isoproterenol (*Science* 133/44/Jan 6/61). JH Sheldon describes natural history of falls in old age (*Brit Med J*, 2/1685/Dec 10/60). CA Swinyard edits symposium on *Role of Erythroblastosis & Kernicterus in Cerebral Palsy* (CC Thomas, Springfield, Ill, 1961, 296pp, 41 illus, $10).

MARCH, 1961

Brain & Behavior: JA Altman & AM Marnseva study electrical responses of acoustic system to consecutive sound stimuli (*Doklady Akad Nauk USSR*, 135/1546/60). F Flach & PF Regan describe *Chemotherapy in Emotional Disorders* (McGraw-Hill, New York 36, 1961, 341pp, $10). E Harms edits conference on psychology of thinking, including theoretical aspects, measurement & creativity (*Ann NY Acad Sci*, 91/1–151/Dec 23/60). A Hoffer & H Osmond approach *The Chemical Basis of Clinical Psychiatry* (CC Thomas, Springfield, Ill, 1961, 277pp, $8.5). WS McCullough edits important & keen conference on *Human Decisions in Complex Systems*, with significant section on the reticular formation (*Ann NY Acad Sci*, 89/715–896/Jan 28/61). E Roberts & Co edit *Inhibition in the Nervous System & Gamma-Aminobutyric Acid*, which could be vice-versa (Pergamon Press, New York, 1960, 591pp, $15). DE Sheer edits discussion on *Electrical Stimulation of the Brain*, considering ways to study problems in neurobehavioral integration (University of Texas Press, Austin, 1961, 524pp, $17.5). P Solomon edits important & revealing symposium on *Sensory Deprivation* (Harvard Univ Press, Cambridge, Mass, 1961, 253pp, $6). J Vernon & TE McGill find that sensory deprivation lowers threshold for pain sensation in humans (*Science* 133/330/Feb 3/61).

Cardiovascular: AA Bagdonas & Co note effects of ischemia & Hypoxia on cardiac conducting system (*Am Heart J*, 61/206/61). RA Caldwell & EW Skipper report on pulseless disease (*Brit Heart J*, 23/53/61). DFJ Halmagy & HJH Colebatch report effects of bretylium tosylate on pulmonary circula-

tion (*Circula Res*, 9/136/61). BF Hoffman & Co discuss concealed cardiac conduction (*Circula Res*, 9/194/61). GM Maxwell & Co describe action of 3-hydroxytyramine (dopamine) on cardiovascular hemodynamics (*Arch Internat Pharmaco*, 129/62/60). JW Remington & CH Baka evaluate blood volume measuring methods (*Circula Res*, 9/60/61). OS Tjong & AP Verhaught study human central & peripheral arterial pressure pulses (*Cardiol* 37/361/61). MW Wheat & TH Burford extend indications for digitalis in surgery (*J Thorac Cardiovasc Surg*, 41/162/61). Y Yoshitoshi & Co report on cardiac lesions in thiamine deficiency (*Jap Heart J*, 2/42/61).

Clinical: WD Alexander & SK Bisset correlate thyroid function with oxygen uptake of human leucocytes (*Quart J Exp Physiol*, 46/46,50/61). HN Antoniades considers the state & transport of insulin in blood (*Endocrin* 68/7/61). GJ Fruchman analyses mobilization of neutrophile (*Trans. NY Acad Sci*, Ser II, 23/30/60). T Hughes calls attention to "growing down," a neglected aspect of ageing (*Pract*, 186/241/61). K Hyllested edits conference on geomedical aspects of multiple sclerosis (Suppl 147, Vol 35, *Acta Psych Neurol Scand*, 1960, 155pp). H Koprowski surveys problems of virus etiology of cancer (*Schweiz Med Wchnschr*, 91/34/Jan 14/61). GW Lohr & HD Waller report on biochemistry & pathogenesis of enzymopenic hemolytic anemia (*Deutsch Med Wchnschr*, 86/87/Jan 13/61). CE Lumsden relates autoimmunity to multiple sclerosis (*Proc Roy Soc Med*, 54/1–48/61). G Mchardy introduces serialized book on *Current Gastroenterology* (*Am J Digest*, 6/28–98/61). F Moya & Co find succinylcholine fails to pass placenta (*Anesth*, 22/1,7,11/61). WD Obrist & Co note diffuse slow EEG abnormalities in old psychiatric patients with low blood pressure (*Neurol*, 11/151/61). FH Smirk reviews recent progress in hypertensive therapy (*Am Heart J*, 61/272/61). WG Smith notes anti-anaphylactic action of ethanolamine & choline (*J Pharm Pharmacol*, 13/1/61). JH Talbot & KL Terplan consider kidneys in gout (*Medicine* 39/405/60). HL Welsh & M Ede recommend cyproheptadine orally for pruritis (*J New Drugs* 1/22/61).

More New Books: EM Blick edits *Trauma in the Aged* with comprehension & skill (McGraw-Hill, New York 36, 1961, 524pp, $16). RD Eastham gives *Biochemical Values in Clinical Medicine: The Results Following Pathological or Physiological Change* (Williams & Wilkins, Baltimore, 1961, 144pp, $3.75). I Galdston reviews *Human Nutrition: Historic & Scientific* (International Universities Press, New York, 1961, 338pp, $6). A Hottinger & H Berger edit *Renal Function & Kidney Disorders in Childhood* (S Karger, Basle, 1960, 571pp, 176 illus, $25). R McKeown tells well about *Pioneers in Mental Health* (Dodd Mead, New York, 1961, 328pp, $3.25). CD Ratnoff discusses *Bleeding Syndromes: A Clinical Manual* (CC Thomas, Springfield, Ill, 1961, 287pp, $8.5). Of prime interest in drug design in FW Schueler's keen *Chemobiodynamics & Drug Design* (McGraw-Hill, New York 36, 1961, 638pp, 110 illus, $1915). NB & AE Taylor edit *The Putnam Medical Dictionary* (GP Putnam's Sons, New York, 1961, 993pp, 100 illus, $5).

APRIL, 1961

A Plethora of Books: AC Aisenberg discusses *The Glycolysis & Respiration of Tumors* (Academic Press, New York 3, 1961, 236pp, $6). HJB Atkins

describes *Tools of Biological Research* (CC Thomas, Springfield, Ill, Vol 1, 1959, 200pp, 200 illus, $8; Vol 2, 1961, 284pp, 180 illus, $10). FC Barter edits *The Clinical Use of Aldosterone Antagonists, as Diagnostic Tools*, in cirrhosis, ascites, cardiac failure & edema (CC Thomas, Springfield, Ill, 1960, 224pp, 112 illus, $5). RW Begg & Co edit *Canadian Cancer Conference* Vol 4 on Biochemistry in Cancer Research, Cell Behavior, Viral carcinogenesis, & chemotherapy (Academic Press, New York 3, 1961, 600pp, $12). J Brachet gives Weizmann Lecture on *The Biological Role of Ribonucleic Acids* (D Van Nostrand, Princeton, NJ, 1960, 144pp, $5). Cold Spring Harbor Symposium in Quantitative Biology, Vol 25, is on *Biological Clocks* (Biological Laboratory, Cold Spring Harbor, NY, 1960, 524pp, $12). FM Foster edits *Evaluation of Drug Therapy* (University of Wisconsin Press, Madison, 1961, 191pp, $4). PS Larson, HB Haag & H Silvette offer comprehensive treatise on *Tobacco: Experimental & Clinical Studies* (Williams & Wilkins, Baltimore, Md, 1961, 944pp, $20). ROK Schade gives keen discussion with atlas on *Gastric Cytology: Principles, Methods & Results* (EC Arnold, London, 1960, 83pp, 85 illus, $8). DE Sheer edits 56 contributors to study of neurobehavioral problems in *Electrical Stimulation of the Brain* (Univ Texas Press, Austin, 1961, 747pp, $17.5). S Shoshan (of Israel) translates collection of undocumented studies on *Evolution of Physiological Functions*, dedicated to L Orbeli by Academy of Sciences USSR (National Science Foundation, Washington, DC, 1960, 162pp, $1.75). L Smith & Co describe *Pain Patterns: Sites & Behavior of Pain in Common Diseases of the Upper Abdomen* (CC Thomas, Springfield, Ill, 1960, 64pp, 112 illus, $12.5). LJ Soffer & Co get into *The Human Adrenal Gland* (Lea & Febiger, Philadelphia 6, Pa, 1961, 600pp, 74 illus, $15). A Towbin analyzes *The Pathology of Cerebral Palsy: The Causes & Underlying Nature of the Disorder* (CC Thomas, Springfield, Ill, 1961, 206pp, $8). A Wernli-Hässig edits *Invalidität & Rehabilitation* (S Karger, Basel & New York, 1961, 184pp, SFr 30). L Zechmeister edits Vol 17, *Progress in the Chemistry of Natural Products* (Lange & Springer, Berlin-Wilmersdorf, 1960, 610pp, $24.5).

Clinical: AM Asatoor & DNS Kerr discuss amines in blood & urine in relation to liver disease (*Clin Chim Acta* 6/149/61). K Baligh & K Lelkes study the tongue in old age (*Gerontol Clin*, 3/38/61). LA French edits symposium in neurosurgery honoring WT Peyton of Minneapolis (*Journ Lancet*, 92/45/61). RJG Fillespie & CC Lucas study effects of ethanol on gastric mucosa, hepatic lipids & cirrhosis (*Canad J Biochem Physiol*, 39/237, 243, 249/61). EF Hartung reviews use of colchicine & analogs in gout (*Arthr Rheumat*, 4/18/61). R Gaunt & Co discuss endocrine pharmacology (*Science* 133/613/Mch 3/61). TW Langfitt discusses mechanism of action of hypertonic urea solutions in reducing intracranial pressure (*Neurol*, 11/196/61). J Lynglye & EF Mogenson study estrogen metabolism in women with liver cirrhosis (*Acta Endocrin*, 36/350/61). J Miller & A Fishman find serotonin antagonist, cyproheptadine, effective in relieving allergic hay-fever, urticaria & headache (*Ann Allergy*, 19/164/61). W Modell discusses clinical pharmacology of digitalis glycosides (*Clin Pharmacol Therap*, 2/177/61). HL Price reviews circulatory actions of general anesthetics (*ibid*, p163). J Rumford makes mortality studies in relation to air pollution (*Amer J Pub Health*, 51/165/61).

HA Selenkow & FM Callaco review clinical pharmacology of antithyroid compounds (*Clin Pharmacol Therap*, 2/191/61 with 197 references). AI Sherman notes radiation resistance in treating cervical cancer (*Am J Roentg*, 85/466/61). CJ Smith & OB Gunn survey vasculitis, mast cells & collagen diseases (*Arthr Rheumat*, 4/1/61). JEC Walker & RE Wells review heat & water exchange in respiratory tract (*Am J Med*, 30/259/61).

Preclinical: E Aaes-Jorgensen discusses essential fatty acids (*Physiol Rev*, 41/1/61). R Fontaine & Co note variations in metabolism of arterial walls in aging (*J Cardiovasc Surg*, 1/236/61). IB Fritz surveys mammalian fatty acid oxidation & synthesis (*Physiol Rev*, 41/52/61). F Heimets & A Herschman consider effects of superimposed electric & magnetic fields on biological systems & electrolytes (*Physics Med Biol*, 5/271/61). LE Hollister reports clinical, biochemical & psychological effects of psilocybin (*Arch Internat Pharmaco*, 130/42/61). J Roberts & W Modell note effect of catecholamines on cardiac rhythmicity (*Circula Res*, 9/171/61). W Salser discusses nongenetic biological information mechanisms (*Persp Biol Med*, 4/177/61). MF Vasiliev correlates nervous system type with vascular reflexes in babies (*Doklady Akad Nauk USSR*, 136/734/61).

MAY, 1961

Basic Material: B Anersson & S Larsson analyse pharmacological control of hunger & thirst (*Pharmacol Rev*, 13/1/61). TD Brock goes into chloramphenicol (*Bact Rev*, 25/32/61). JL Butterfield & JA Richardson show guanethidine increases cardiac contractility with depletion of cardiac norepinephrine content (*Proc Soc Exp Biol Med*, 106/259/61). J Dobbing surveys the "blood-brain barrier" (*Physiol Rev*, 41/130/61). H Duner & Co ask if urinary excretion of imidazole acetate reflects histamine metabolism in humans (*Acta Physiol Scand*, 51/41/61). MV Edds edits *Macromolecular Complexes* (Ronald Press, New York 10, 1961, 269pp, 107 illus, $7). N Emmelin discusses pharmacological denervation (*Pharmacol Rev*, 13/17/61). AP Fishman analyses regulation of pulmonary circulation by respiratory gases (*Physiol Rev*, 41/214/61). WH Fishman neatly summarizes *Chemistry of Drug Metabolism* (CC Thomas, Springfield, Ill., 1961, 252pp, $6). SJ Freedman & Co sympose on sensory deprivation with facts hunting theory (*J Nerv Ment Dis*, 132/17–44/61). WJ Gardner suggests blood-brain barrier expresses lack of interstitial spaces in ectodermal tissue (*Persp Biol Med*, 4/169/61). W Grab considers pharmacological aspects of wines & spirits (*Arzneimittelforschung* 11/73/61). G Hollunger, in studying metabolism of lidocaine, describes amide hydrolyzing microsomal enzyme (*Acta Pharmacol Toxicol* 17/356, 365, 373, 384/61). J Holubar & J Ipser edit *Electrophysiological Methods in Biological Research* (Czechoslovak Academy of Sciences, Prague, 1960, 515pp). S Jablowski & RM Leonard compile informative Russian Drug Index (*National Library of Medicine*, Washington 25, DC, 1961, 109pp, $0.6). DE Koshland edits *Protein Structure & Function* (Brookhaven Nat Lab, Upton, NY, 1961, 266pp, $2.5). VG Longo offers *Effect of Drugs on the Electrical Activity of Rabbit Brains*, an EEG atlas (D Van Nostrand, Princeton, NJ, 1961, 605pp, 495 illus, $20). EW Poole studies relation of nervous activity to respiratory cycle (*Nature*, 189/579/Feb 18/61). JH &

DMJ Quastel well analyse 524 reports on *The Chemistry of Brain Metabolism in Health & Disease* (CC Thomas, Springfield, Ill, 1961, 182pp, $5). RC Read & JA Vick note cholinergic-like effects of hypertonic solutions (*Am J Physiol*, 200/233/61). GK Smelser edits good symposium of Congress of Anatomists on *The Structure of the Eye* (Academic Press, New York, 1961, 570pp, $15). H Winterstein considers intracranial chemoreceptors (*Pharmacol Rev*, 13/71/61).

Clinical: WC Alvarez sympathetically describes *Minds That Came Back*, with annotated bibliography of 350 autobiographical accounts (JB Lippincott, Philadelphia, 1961, 384pp, $6). P Bailey riggs an interview on ego ideals, with illustrations & plea for religious sympathy (*Persp Biol Med*, 4/199/61). J Garland & J Stokes edit *The Choice of a Medical Career: Essays on the Fields of Medicine* (JB Lippincott, Phila, 1961, 241pp, $5). F Glenn edits symposium on circulatory system in surgery (*Surg Clin N Amer*, 41/265–532/61). I Hieger discusses *Carcinogenesis* (Academic Press, London SW 1, 1961, 138pp, 35S). DS Howell & Co advise against use of polyvinyl alcohol as a pliable prothesis in human surgery because it induces mineral deposition (*Proc Soc Exp Biol Med*, 106/317/61). RA Kuhn offers *New Hope for Stroke Victims* (Appleton-Century-Croft, New York 1, 1961, 206pp, $4). HD Levine advises on *Cardiac Emergencies & Related Disorders* (Appleton-Century, New York 1, 1961, 368pp, 45pp illust, $12). DW Mulder & Co discuss neuropathies in diabetes mellitus (*Neurol* 11/275/61). FC Reubi opens Ciba international symposium on *Essential Hypertension*, with neat review by IH Page of his mosaic theory of its etiology (Springer, Berlin, 1960, 398pp, DM 56). RP Schmidt introduces conference on vascular disease of the brain (*Neurol*, Vol 11, No 4, Pt 2, 176pp). H Selye & Co show thallium acetate potentiates dihydrotachysterol calcinosis in aorta, kidney & injured skin (*Proc Soc Exp Biol Med*, 106/408/61). RL Swank considers a *Biochemical Basis for Multiple Sclerosis* (CC Thomas, Springfield, Ill, 1961, 100pp, 23 illust, $5). Note symposium on effective medical writing by masters therein (*New Physician*, March, 1961). GEW Solstenholme & CM O'Conner edit *Haemopoieses: Cell Production & Its Regulation* (Little Brown, Boston, 1961, 490pp, $11).

General: I Asmov explains *Words from the Myths* (Houghton Mifflin, Boston, 1961, 225pp, $3). WO Fenn introduces symposium on biological communications, dealing with theory, structure, function & management (*Biol Abst*, Vol 36, No 7, svii–xxvi, April 1/61). W Gerlach considers J Kepler (1571–1630) in relation to ethics of research (*Naturwisch*, 48/85/61). OS Hayward tells well of Nathan Smith (1782–1829), great teacher (*Conn Med J*, 24/553/60; *New Eng J Med*, 263/1235, 1288/Dec 15, 22/60). EB Hague opens discussion on development of microscopy (*New York State J Med*, 61/430–454/61). AV Hill well discusses *The Ethical Dilemma of Science* & other matters (Rockefeller Inst Press, New York, 1960, 395pp, $7.5). R Kervan tells of *Laennec: His Life & Times* (Pergamon Press, London, 1960, 223pp, 21S). See LL Strauss' review of CP Snow's *Science & Government* (Harvard Press, 1961, 88pp, $2.5) in Apr 2 *NY Times* Book Section.

CHAUNCEY D. LEAKE